

DEVELOPING CHALLENGE MODELS AND VACCINE EFFICACY TESTS
AGAINST *Streptococcus iniae* FOR TWO ORNAMENTAL CYPRINID FISH: THE
RED-TAIL BLACK SHARK (*Epalzeorhynchus bicolor*) AND THE RAINBOW
SHARK (*E. erythrurus*)

By

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To my parents Francesca and Salvatore, my aunt Marilena, my uncle Ennio, and my wife
Juli-Anne. Thank you for your love and encouragement.

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AGAINST *Streptococcus iniae* FOR TWO ORNAMENTAL CYPRINID FISH: THE
RED-TAIL BLACK SHARK (*Epalzeorhynchus bicolor*) AND THE RAINBOW
SHARK (*E. erythrurus*)

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The purpose of our study was to test three autogenous vaccine formulations (a) basic formula; b) basic formula with aluminum salt adjuvant; c) basic formula with oil adjuvant) developed by NOVARTIS-Aqua Health LTD against *Streptococcus iniae*, for two ornamental fishes: *Epalzeorhynchus bicolor* (red-tail black shark) (RTB shark) and *E. erythrurus* (rainbow shark). These two species of fish were chosen because they are considered economically important for the Florida ornamental industry, and they are very susceptible to streptococcal diseases.

We first developed a challenge model against *S. iniae* for the RTB shark, and then tested it on rainbow sharks. The protection afforded by the three formulations and different routes of applications (intracoelomic injection, bath, oral) were then tested on RTB sharks. These tests showed that only the injection route of administration and the two formulations with adjuvant were effective in protecting the fish.

The three vaccine formulations were then tested on rainbow sharks, and differences in vaccine efficacy among juvenile and adult groups of both RTB and rainbow shark were investigated. Results of these experiments showed that the vaccine conferred a higher protection level to RTB than to rainbow sharks. Farmers have reported that the blue shark (a color variant of the rainbow shark) is more resistant to *S. iniae*. Our study confirmed this.

In another set of experiments, the immunostimulatory capability of beta-glucans and nucleotides were tested. Both compounds decreased the mortality of RTB sharks challenged with *S. iniae* compared to the controls. The final step of our study was to determine if the vaccines were effective in protecting RTB broodstock fish against *S. iniae* infections resulting from the stress caused by artificial spawning. Results showed that all three vaccines efficiently protected fish after artificial spawning.

Our study showed the efficacy of the two vaccine formulations with adjuvants against *S. iniae* and the efficacy of two immunostimulant compounds, beta-glucans and nucleotides for two species of ornamental fish. Furthermore, these challenge and vaccine efficacy models can be used as templates in future studies with other species of ornamental fish and other bacterial diseases.

CHAPTER 1 INTRODUCTION

The purpose of our study was to test three autogenous vaccine formulations (a basic formula, and formulae with the addition of aluminum salt or oil as adjuvants) against *Streptococcus iniae*, for two ornamental fish species, *Epalzeorhynchus bicolor* (red-tail black shark) (RTB shark) and *E. erythrurus* (rainbow shark). We chose these two species of fish because they are considered economically important for the Florida ornamental fish industry, and because they are very susceptible to streptococcal diseases. We chose *Streptococcus iniae* infection because is an important disease in the aquaculture industry, and has been reported worldwide causing up to 75% mortality.

The first step in our study was to build a biosecure laboratory (Chapter 8). A challenge model against *S. iniae* was developed for both fish species (Chapter 9). We tested the protection offered by the three formulations and different routes of applications on juveniles and adults of both species (Chapters 10-12). We then examined differences in vaccine efficacy between rainbow and blue sharks (a selected phenotypic variant of rainbow shark) (Chapter 13). Immunostimulatory capabilities of beta-glucans and nucleotides were tested on RTB shark (Chapter 14). Then, we examined whether the vaccines were effective in protecting RTB broodstock against *S. iniae* infections resulting from the stress caused by artificial spawning (Chapter 15). Agglutination and ELISA tests were run to detect shark antibodies and specific anti-*S. iniae* shark antibody (Chapter 16).

CHAPTER 2
RED TAIL BLACK (*Epalzeorhynchus bicolor*) AND THE RAINBOW SHARK (*E. erythrurus*) BIOLOGY AND AQUACULTURE

2.1. Biology

Red-tail black shark (*Epalzeorhynchus bicolor*) and rainbow shark (*E. erythrurus*) are important economic species for the ornamental fish industry. The name “shark” may lead to the misunderstanding that these fish belong to the Elasmobranchs, real sharks and rays. In reality these fish belong to the class Actinopterygii, family Cyprinidae. This family includes the common carp (*Cyprinus carpio*) and danio (*Danio* spp.). These fish are called “shark” only for marketing purposes and because the dorsal fin is vaguely reminiscent of the fin of a real shark.

Epalzeorhynchus bicolor has a black body with a red tail, while *E. erythrurus* has a greenish-brown body with a large black spot at the base of the caudal fin; and the dorsal, anal, and pelvic fins are red. Both species attain a maximum total length (TL) of 15 cm (Axelrod and Burgess, 1997). These fish are benthopelagic, tropical freshwater fish. Their natural environment is water with a pH range of 6.5-7.5, a temperature of 22-27°C, and a hardness of 15 dH. Sharks are very sensitive to high concentrations (>0.04 ppm) of NO₂⁻. The diet of the shark includes algae, periphyton, plant matter, worms, crustaceans, insects, and zooplankton (Yang and Winterbottom, 1998). Sharks are very aggressive to each other, and for this reason, it is difficult to keep individuals in the same tank. A higher number of individuals sometimes helps to decrease aggressive behavior.

These species originate from Southeast Asia, particularly the Chao Phraya (Thailand) and Mekong basins. It is not clear if *E. bicolor* still exists in the wild (Baillie et al., 2004). Presently, this species has been placed in the IUCN Red List in the category of extinct in the wild. The reasons for its decline in the wild are not clear. The aquarium fish trade has been accused of driving the species to extinction by overfishing, but no evidence is documented (Kottelat and Whitten, 1996). Furthermore, most of the sharks sold in the ornamental industry are farm raised and not caught in the wild. Habitat modification may be a more likely cause of their population decline. In the 1970s many dams were constructed in Thailand, and these probably severely impacted the natural ecosystems of several large basins in the region. Furthermore, large swamps have been drained, decreasing the natural habitat of this species (Dai and Yang, 2003).

2.2. Aquaculture

Red-tail black and rainbow sharks can be raised and spawned commercially on farms. Broodstock can be reared in ponds and in vats. The advantage of rearing broodstock in vats is that they can be conditioned all year around. In ponds, fish are conditioned naturally; and spawn in Florida only between May and September. The indoor conditioning period lasts 6 weeks with fish kept at 28°C and 16 h light. Broodstock are then artificially spawned by induction with hormones (Rottmann et al., 1991a,b). Each broodstock can be used for spawning up to 16 times in a period of 3 years (personal communication by farmers). In captivity, females become mature at 1.5 years old, corresponding to a size of 10-13 cm. They can produce several thousand eggs.

Eggs hatch after 24-48 h at a temperature of 25-26°C. The larvae and juveniles are then raised in indoor vats for 2-4 weeks at 25-26°C before being moved to outdoor ponds.

In the third and fourth weeks, when fish are 4-8 mm long, they begin to be pigmented.

After 5-8 months, when the juveniles reach 2.5-5 cm, they are harvested and sold to the pet market. Larger fish are also sold, but the market demand is less (personal communication by farmers).

CHAPTER 3 STREPTOCOCCAL MICROBIOLOGY

Streptococci are gram-positive, not motile, and catalase-negative bacteria. In actively spreading lesions within host tissues, diplococcal and individual coccal forms are common; in artificial media and purulent exudate, chains are more common. The length of the chain tends to be inversely related to the adequacy of the culture medium. Factors that tend to promote exaggerated chaining include conditions that impair growth (such as unfavorable media, cold temperature, or antimicrobial agents); and also the presence of antibody that reacts with cell wall antigens (Woo and Bruno, 1998). All streptococci are lactic-acid bacteria, and derive their energy primarily from fermenting sugars, regardless if they are grown aerobically or anaerobically. The accumulation of lactic acid in media with high glucose content can limit bacterial growth if the pH is not buffered or corrected.

Several kinds of streptococci can be isolated from humans and other animals. The first proposed classification of streptococci was based on the description of the type of hemolytic reactions observed on blood agar plates. In this classification the bacteria are divided into three groups: alpha, beta and gamma. A narrow zone of hemolysis surrounds alpha-hemolytic streptococcal colonies, with unhemolyzed RBCs in an inner zone, and complete hemolysis in an outer zone surrounding the colonies. The mechanism responsible for sparing some red blood cells is not known. Frequently it is possible to observe a green discoloration of the colonies due to the formation of an unidentified reductant of hemoglobin. The grade of discoloration depends on the type of blood in the

medium, and on the incubation time. Because of this feature, this streptococcal group is also called the viridans group. Most of the streptococcal strains that cause human disease belong to Group A. Beta-hemolytic streptococci have an outer capsule, and produce a wide clear zone of complete hemolysis in which no red cells are visible upon microscopic examination. These bacteria can release two types of beta-hemolysin enzyme: streptolysin O and streptolysin S. Streptolysin O is destroyed by atmospheric oxygen, and is therefore demonstrable only in deep colonies. Streptolysin S is stable in contact with atmospheric oxygen, and is responsible for surface colony hemolysis. Beta hemolytic streptococci are differentiated into a number of immunologic groups designated by the letters A through O; in each group, different streptococcal types are present. The groups are characterized by different external carbohydrates that act as antigens, while specific antigen proteins characterize the types. Precipitin tests (Lancefield) and aggregation reactions (Griffith) showed this difference in antigens. *Streptococcus iniae* is included in the beta-hemolytic streptococcal group.

Gamma-streptococci produce no hemolysis either on the surface or within the agar. This classification based on the hemolysis pattern is not very satisfactory (Woo and Bruno, 1998) for the following reasons:

- Many species classified as beta-hemolytic are in fact not hemolytic (certain members of the antigenic groups B, C, D, H, K, O and all group N).
- Most streptococci found in the gastrointestinal tract are nonhemolytic, but some strains produce beta-hemolysis (group D).
- Some strains classified as nonhemolytic after 24 h incubation may show alpha-hemolysis if incubated for an additional 24-48 h.

CHAPTER 4 STREPTOCOCCOSIS IN FISH

4.1 Pathology

Streptococcal infections in fish result in septicemic diseases that have been reported worldwide. They cause an estimated \$150 million in annual losses of fish production (Shoemaker and Klesius, 1997). Streptococcosis can cause up to 75% mortality in closed culture systems (Perera et al. 1994) and up to 50% in ponds (Eldar et al. 1997). It is one of the most important diseases in South Africa, and Japan (Ferguson et al. 1994), in yellowtail farming (Bang and Sung-Hee, 1993); and is among the most important summer diseases in Italian and Israeli trout farming (Bercovier et al. 1997). Streptococcosis can affect wild and farmed fish in both fresh and saltwater. Case reports in saltwater fish include yellowtail, *Seriola quinqueradiata* (Aoki et al. 1990; Ikeda and Minami, 1982; Park et al. 1987; Sako, 1998), turbot, *Scophthalmus maximus* (Romalde et al. 2000), Japanese flounder, *Paralichthys olivaceus* (Nakatsugawa, 1983), mullet, *Mugil cephalus* (Bunch and Bejerano, 1997), red drums, *Sciaenops ocellatus* (Eldar et al. 1999), striped piggy, *Pomadasys stridens*, and variegated lizardfish, *Synodus variegatus* (Colomi et al. 2002). Case reports in freshwater fish include tilapia, *Oreochromis* spp. (Shoemaker et al. 2001), Mozambique tilapia, *Tilapia mossambica* (Ming-Chen et al. 1985), Nile tilapia, *Tilapia nilotica* (Kitao et al. 1981), hybrid tilapia, *Tilapia aurea* x *T. nilotica* (Perera et al. 1997; Perera et al. 1998), sunshine bass, *Morone saxatilis* male x *M. chrysops* female (Stoffregen et al. 1996), hybrid striped bass, *M. chrysops* male x *M. saxatilis* female (Shoemaker et al. 2001), rainbow trout, *Oncorhynchus mykiss* (Eldar et

al. 1995a; Kitao et al. 1981), eel, *Anguilla japonica* (Kusuda et al. 1978), ayu, *Plecoglossus altivelis* (Kitao et al. 1981; Ugajin, 1981), snakeheads, *Ophicephalus striatus* (Lio-Po et al. 1998), walking catfish, *Clarias batrachus* (Lio-Po et al. 1998), zebra danio, *Danio rerio*, and pearl danio, *D. albolineatus* (Ferguson et al. 1994), clown loaches, *Botia macracanthus*, rainbow shark, *E. erythrurus*, red-tailed black sharks, *E. bicolor*, rosy barbs, *Barbus conchoni* (Russo data not published), tetras, *Hyphessobrycon* sp., and African cichlids of the genera *Nimbochromis* and *Pelvicachromis* (Yanong and Floyd, 2002). Cases of streptococcosis in wild fish are reported in several publications (Baya et al. 1990; Colorni et al. 2002; Zlotkin et al. 1998). The possibility of streptococcal outbreaks is increased if fish are stressed, as happens with non-optimal water temperature, low dissolved oxygen, high nitrite levels, and high culture densities (Bunch and Bejerano, 1997; Perera et al. 1997).

Several species of streptococci have been isolated from fish. Alpha-hemolytic streptococci generally cause granulomatous inflammation; whereas beta and gamma-hemolytic streptococci cause systemic infection localized in various organs (mainly spleen, kidney, brain, liver, and eye), with the presence of granulomas in some cases (Ferguson et al. 1994; Chang and Plumb, 1996). An alpha-hemolytic streptococcus was isolated from hybrid tilapia (Al-Harbi, 1994) showing erratic and whirling swimming, exophthalmia, hemorrhagic areas on the body surface, and the presence of fluid in the abdominal cavity as the main clinical signs. Another alpha-hemolytic streptococcus, with characteristics similar to *S. faecalis* and *S. faecium*, was isolated from *Siganus canaliculatus*, white spotted rabbitfish, in Asia (Foo et al. 1985). Affected fish presented whirling swimming, blindness, and dark coloration, suggesting that the bacteremia

affected the central nervous system. No external ulcerations and hemorrhages or other signs were observed. *Streptococcus difficile* and *S. shiloi* were first isolated in Israel from tilapia and trout with meningoencephalitis (Eldar, et al. 1994; 1995b). Two efficacious vaccines against *S. difficile*, a formalin-killed and a bacterial extract based, have been developed (Eldar et al. 1995b). Other streptococci isolated from fish are *S. agalactiae* isolated from seabream and wild mullet (Evans et al. 2002), and *S. parauberis* isolated from turbot (Domenech et al. 1996; Romalde et al. 2000).

Streptococcus iniae is the most commonly isolated streptococcus from fish (Bromage et al. 1999; Eldar et al. 1999; Fiske and Johnson, 1998; Perera et al. 1994; 1997; Sako, 1998). *Streptococcus iniae* causes a systemic disease characterized by histological and external pathology and by characteristic fish behavioral changes. The disease is associated with a strong inflammatory response by the host where acute necrotizing reactions and systemic neutrophil and macrophage exudation can be observed in several organs and tissues in conjunction with the presence of high numbers of gram-positive cocci (Ferguson et al. 1994; Neely et al. 2002).

Externally, streptococcosis-induced lesions include pronounced congestion and hemorrhages, especially around the base of the pectoral fins and over the heart. Exophthalmia, corneal opacity, and intra-ocular and periorbital hemorrhages can also be observed (Ferguson et al. 1994; Liu et al. 1990; Perera et al. 1998).

The main organs affected by *S. iniae* are the brain, spleen, and posterior kidney. Pathological changes associated with the brain and nervous system are typical of streptococcosis infections. A common presentation of *S. iniae* infection is meningoencephalitis (Camus, 2002; Ferguson et al. 1994; Neely et al. 2002). Meningeal

granulomas and massive cellular infiltration can be observed (Eldar et al. 1995a; Perera et al. 1998). Spleen and posterior kidney are common targets because of their high capability to trap bacteria. In the later stages of infection, there is an increase in size, expressed as percent body weight, of the spleen and kidney (Ikeda and Minami, 1982). Analysis of the spleen can reveal congestion and fibrinoid necrosis of the ellipsoidal sheaths with loss of reticular macrophages. Bacteria can be widely scattered throughout the parenchyma of the spleen, but can also be observed within macrophages (Ferguson et al. 1994; Perera et al. 1998). Microscopic examination of the posterior kidney can reveal severe congestion of the renal parenchyma, and pronounced degeneration and necrosis of many tubules (Ferguson et al. 1994; Perera et al. 1998).

A large number of bacteria can be observed in the pericardial area, especially within the hemorrhagic musculature associated with the pectoral girdle (Ferguson et al. 1994; Liu et al. 1990; Perera et al. 1998). Other organs that can be affected are the liver, where sub-capsular petechial hemorrhages and granulomas have been observed; and the intestinal tract, where bacteria have been observed in the lamina propria; but not in the mucosa (Ferguson et al. 1994; Perera et al. 1998). An elevated number of bacteria can be observed within blood vessels, especially in the capillary network of the gills, cranial meninges, retina, and choroidal rete. The presence of bacteria in the capillaries caused occasional thrombosis (Ferguson et al. 1994; Perera et al. 1998). *Streptococcus iniae* can cause typical behavioral changes such as a swimming spinning pattern (Eldar et al. 1995a; Perera et al. 1998).

In our research, fish infected with *S. iniae* showed similar external signs and histological findings (Chapter 9) as the ones described in the previous paragraphs.

However, differently from the studies of Ferguson et al. (1994) and Perera et al. (1998), during histological examination no granulomas were observed.

One concern with *Streptococcus iniae* is the possible transmission of the bacteria from fish to humans. Few cases have been reported. Apparently the probability of such transmission is low. It is a concern mainly with immunocompromised individuals (Goh et al. 1998; Lau et al. 2003; Weinstein et al. 1996; 1997). However, recent reports in China suggest a possible higher risk of *S. iniae* infections in Asian populations due to their habit of cooking and eating fresh fish (Lau et al. 2003). Pulse-field gel electrophoresis (PFGE) has showed that *S. iniae* strains that cause disease in fish are genetically diverse from commensal strains isolated from nondiseased fish (Fuller et al. 2001; Weinstein et al. 1997).

4.2. Host-Pathogen Interactions

Streptococcus iniae, like streptococci belonging to the Group B (GBS), shows a tropism for the brain. This tropism is one of the main causes of death. The invasion rate of *S. iniae* in the different organs has been experimentally observed in yellowtail (Kusuda and Kawai, 1982; Kusuda and Kimura, 1978; Sako, 1998). In the first 2 days after challenge, there is an increase in the bacterial count of blood, spleen, and kidney; followed by a decrease or a constant count value. In contrast, 2-3 days after challenge there is a constant increase of bacterial count in the brain until the onset of mortality. The exact mechanism used by *S. iniae* to cross the blood-brain barrier (BBB) is still not well understood. It involves production of the cytolysin streptolysin S (SLS) required for local tissue necrosis (Camus, 2002; Fuller et al. 2002; Kimura and Kusuda, 1979; Kusuda and Hamaguchi, 1989; Romalde et al. 2000; Sakai et al. 1992). *Streptococcus iniae*, as GBS,

could elicit its own endocytotic uptake from the microvascular endothelial cells and so pass the BBB (Nizet and Rubens, 2000). Otherwise, as observed with *S. suis*, *S. iniae* could adhere and damage the microvascular endothelial cells through the action of SLS, and then proceed from the circulation to the central nervous system (Charland et al. 2000).

The pathogenesis mechanisms of GBS have been studied in detail and resemble those of systemic infection with *S. iniae*. For this reason, the GBS pathogenesis mechanisms may be very useful in understanding the possible mechanisms used by *S. iniae* (Miller and Neely, 2004). Invasion of the brain microvascular endothelial cells by GBS causes an evident cellular injury and increased cell permeability because of the cytotoxicity activity of the beta-hemolysin/cytolysin (b-h/c) that causes formation of pores on the epithelial tissue (Nizet et al. 1997; Ring et al. 2002a). Streptococcal mutants with a hyperhemolytic phenotype give evidence of the involvement of b-h/c on host tissue damage. Histological examination of animals infected with these mutants shows higher degree of necrotic tissue with these strains of bacteria (Ring et al. 2002a). The b-h/c is also important for penetrating the BBB. In animals infected with mutants lacking b-h/c, fewer bacteria penetrate the BBB, and the mortality rate and bacterial numbers in brain increase with increased levels of b-h/c activity (Nizet et al. 1996). In addition, b-h/c has proinflammatory, proapoptotic, and proinvasive properties that could contribute to disease pathogenesis. Mice b-h/c production is associated with increased local and systemic levels of proinflammatory cytokines, such as IL-6 and IL-1 released by injured endothelial cells (Liu and Nizet, 2004). The proinflammatory mediators, such as IL-8, GRO-alpha, ICAM-1, and GM-CSF, are chemotactic factors; promote transendothelial

migration, and stimulate the respiratory burst, degranulation, and adherence of leukocytes and neutrophils. The development of inflammatory exudates then promotes further damage of the BBB and stimulation of apoptosis of neurons and other cells (Doran et al. 2003; Nizet et al. 1997). Leukocytic infiltration into the cerebrospinal fluid is a major clinical diagnostic marker for acute bacterial meningitis.

Group B Streptococci may also induce epithelial damage indirectly by altering host cell processes, such as the cell peroxidase-H₂O₂ system that can damage adjacent cells by producing oxygen radicals. The observed hypotension in septic shock could also be caused by an excess production of nitric oxide (NO) stimulated by the GBS (Ring et al. 2000; 2002b). The GBS can also modify the arachidonic acid metabolism favoring the production of prostaglandin E₂ (Sbarra et al. 1987). Bacteria replication within CNS may then provoke an overwhelming host inflammatory response that, in combination with the disruption of the BBB, could be fatal (Doran et al. 2003). *Streptococcus iniae*, as with GBS, could also secrete other factors that break down the extracellular matrix helping to spread the bacteria, or that inactivate the inflammatory mediators, such as C5a, thus decreasing recruitment, and the opsonophagocytic capacity of neutrophils (Cheng et al. 2002; Liu and Nizet, 2004).

The polysaccharide capsule of *Streptococcus* protects the bacteria by decreasing adhesion of immune cell receptors to the bacteria, (Tamura et al. 1994). Sialic acid residues on the capsule can inhibit activation of the complement alternative pathway, thereby interfering with the opsonophagocytic killing mechanism of the neutrophils, and macrophages. The polysaccharide capsule is also important for the survival of *S. iniae* inside macrophages, as suggested by the observation of live bacteria inside blood

macrophages. The ability of *S. iniae* to survive in macrophages may be useful in establishing a systemic bacteremia and for crossing the BBB within macrophages migrating in the CNS. The ability to overcome the immune response of macrophages is one characteristic that allows *S. iniae* to establish an infection. Another defense mechanism used by *S. iniae* is inhibiting apoptosis and increasing necrosis of natural cytotoxic cells in fish (NCC) (Doran et al. 2003; Fuller et al. 2001; Martin et al. 1992; Taylor et al. 2001; Zlotkin et al. 2003).

Data collected in recent research shows that recognition of GBS by immune cells and their later activation involves Toll-like receptors (TLRs). The same receptors may also be involved in recognition of *S. iniae* (Henneke et al. 2001; 2002).

Both the innate and acquired parts of the immune system are involved during *Streptococcus* infections. Nonspecific cytotoxic cells (NCC) are part of the nonspecific immune system, and play an important role in fish during bacterial infections (Evans et al. 1998; 2000a; Taylor et al. 2001; Weinstein et al. 1997). Nonspecific cytotoxic cells have cytotoxic action. They release cytokines that increase phagocytosis by macrophages, activate antigen-processing cells, and augment the inflammatory response. Nonspecific cytotoxic cells become biochemically activated; this increases their cytotoxicity after the first encounter with a stressor agent. These responses are different from those occurring during normal homeostatic regulation, where the encounter of NCC with a target cell represented by a parasite or a tumor cell, causes apoptosis of the NCC. Prokaryotic DNA can activate the cytotoxicity mechanism in NCC, and it is involved in the interactions of B-cells, macrophages, and dendritic cells. All these cells can recognize prokaryotic DNA because it is nonmethylated. Treatment of NCC with eucaryotic DNA has no effect on

cytotoxicity. Also treatment of bacterial DNA with DNAase or methylation of all cytosine bases, abrogates the cytotoxic enhancement (Oumona et al. 2002). These observations indicate that DNA binding receptors on NCC and other immune cells have a mechanism for preferring certain nucleotides. This mechanism should be similar to MHC recognition and presentation of peptides, where certain amino acids serve to anchor the peptide to the MHC antigen. Studies using synthetic oligonucleotides shown that cytotoxic activation is enhanced by bacterial DNA fragments containing –GACGTT- and –GTCGTT- sequences. These results also suggest that binding and internalization of bacterial DNA is required for initiation signaling and cytokine release. Recognition of bacterial DNA by NCC appears to be determined by a combination of sequence and conformational structure. This mechanism may be similar to the recognition of antigenic determinants by T and B-cell receptors. Apparently, different subsets of NCC could exist with different specificities for bacterial DNA receptors, or individual NCC could contain receptors with different specificities (Babiuk et al. 2000; Krieg, 2000; Scheule, 2000). These results are important to better design and understand the mechanism of action of DNA vaccines.

Streptococcus iniae inhibits apoptosis and increases necrosis of NCC. The advantage of a decreased number of apoptotic cells is that apoptotic cells are phagocytosed and do not initiate an inflammatory response. In contrast, cells undergoing necrosis are eventually lysed, and release several compounds that will activate the inflammatory response. Another effect is increased antigen processing which increases cellular recruitment, and thus increases antibody production and cell-mediated immune responses. One mechanism responsible for the decreased apoptotic cell rate is observed in

some strains of *S. iniae* where phosphatidylserine expression is inhibited in the NCC outer membrane. Recognition of phosphatidylserine by macrophages is needed for initiating the phagocytosis process (Taylor et al. 2001).

The possibility that protection against bacteria in fish mainly involves the cellular immune system of the non-specific defense system could explain the results of several vaccine studies (Eldar et al. 1995b; Evans et al. 2000b; Lillehaug et al. 1993; Taylor et al. 2001). They observed no correlation between antibody titers and protection. In other studies the protection given by vaccination lasted only for a few months (Eldar et al. 1995b; Hortvitz et al. 1997; Iida et al. 1981).

Nevertheless, the humoral part of the immune system is important in the host defense mechanism. In yellowtail (Kusuda and Tanaka, 1988) specific antibodies increased the phagocytic activity and index of macrophages. This effect could not be duplicated if complement alone was added to cell culture. However with both antibody and complement, the phagocytic activity and index were significant higher. These results suggest that during bacterial infections in fish, activation of the classical pathway of the complement by antigen-antibody reaction is an important mechanism of defense.

Antibody production appears to be stimulated more by toxins released by *Streptococcus* than by the bacteria itself (Kusuda and Takagi, 1983). These results highlight the possibility that toxoid vaccines may work better than bacterial vaccines.

4.3. Antibiotics Treatments and Vaccines

The antibiotic used most to treat *Streptococcus* spp. infections is erythromycin (Noga, 1996; Stoffregen et al. 1996; Treves-Brown, 2000). Erythromycin inhibits the protein synthesis and elongation of bacteria by binding the 23S rRNA molecule of the

50S subunit of the bacterial ribosome (eukaryotes do not have the 50S ribosomal subunit) (Treves-Brown, 2000). Some erythromycin resistant strains of bacteria present mutations in components of the 50S ribosomal subunit, and for this reason the antibiotic fails to bind the ribosome. The non ionized form of the drug is considerably more permeable to cells, and this probably explains the increased antimicrobial activity that is observed in alkaline pH. The oral recommended dose for fish is 25-50 mg erythromycin/kg fish/day for 4-7 days. The injectable dose is 10 to 20 mg/kg fish for 14 days.

Oxytetracycline was tested as a possible treatment for *S. iniae*, but efficacy varied among experiments. Oxytetracycline inhibits the bacterial protein synthesis by blocking the attachment of the complex tRNA-amino acid to the ribosome. Oxytetracycline can less likely inhibit the protein synthesis in the host, because eukaryotic cells do not have a tetracycline uptake mechanism (Treves-Brown, 2000). In blue tilapia infected with *S. iniae*, administration for 14 days of medicated feed containing oxytetracycline at levels between 50 and 100 mg OTC/kg fish/d fed at 2% body weight/day increased the survival rate between 45 and 98% (Darwish et al. 2002). The minimum inhibitory concentration (MIC) of oxytetracycline against multiple *S. iniae* isolates indicated a general sensitivity at concentrations of 0.25-0.50 µg/mL. Completely different results were observed in hybrid striped bass infected with *S. iniae* (Stoffregen et al. 1996). In this case, treatment with Terramycin for Fish® (Pfizer, Inc., Connecticut, USA) at doses of 82.7 mg drug/kg fish per day for 10 days was unsuccessful. The different results could be explained partly by the higher resistance of this bacterial strain to oxytetracycline with a MIC of 2.0 µg/mL. High resistance of *Streptococcus* sp. to tetracycline antibiotics was also observed in bacteria isolated from yellowtail (Aoki et al. 1990).

Amoxicillin has been used to treat infections from *S. iniae* with a range of dose of 30–120 mg/ kg fish body weight administered for 8 consecutive days (Darwish and Ismaeil, 2003). Amoxicillin, as other penicillins, interferes with the bacteria cell wall synthesis preventing the development of the peptidoglycan layer (Treves-Brown, 2000).

Another antibiotic tested as a possible treatment for streptococcosis in one study is josamycin (Takemaru and Kusuda, 1988). Josamycin inhibits the protein synthesis by binding the 23S rRNA or 50S bacterial ribosomal subunit (Treves-Brown, 2000). In the study of Takemaru and Kusuda (1988), the MICs of josamycin, erythromycin, and spiramycin were compared. Observed josamycin MIC values ranged between 0.10 and 0.39 µg/mL. They were higher than the values recorded for spiramycin, but lower than the ones for erythromycin. The development of resistance of the bacteria to the antibiotics after 9 successive subcultivations was 4-fold higher in MIC value for josamycin and 8-fold higher for the other two antibiotics. These results support josamycin as a possible treatment against streptococcosis.

Florfenicol is one of the newest antibiotics on the market, and it has been used against several bacteria such as *Aeromonas salmonicida*, *Yersinia ruckeri*, *Flavobacterium psychrophilum*, *Vibrio salmonicida* (Michel et al. 2003; Nordmo et al. 1998), and *Edwardsiella ictaluri* (Gaikowski et al. 2003). This antibiotic has also been tested in ornamental fish (Yanong et al. 2002). Florfenicol binds reversibly the large ribosomal subunit of bacteria and eukaryotes, and it also binds the peptidyl transferase enzyme inhibiting the transfer of the growing polypeptide to the next amino acid occupying the "acceptor" site. However, florfenicol binds easier the bacterial ribosome than the eukaryotic ribosome (Treves-Brown, 2000). Florfenicol may also be used to treat

Streptococcus infections, as is reported by the label of Aquaflor (active ingredient Florfenicol 500 mg/g, Schering Plough, Union, New Jersey) and from the results of some investigations (Yanong et al. 2004).

Several vaccines have been developed against streptococci, mainly for tilapia and rainbow trout. Protection levels given by the vaccines varied among experiments and vaccine formulations (Akhlaghi et al. 1996; Bachrach et al. 2001; Eldar et al. 1995b; Eldar et al. 1997; Klesius et al. 2000; Klesius et al. 2001; Lim and Klesius 2001a; Palacios et al. 1993; Sakai et al. 1987; Shelby et al. 2002a). An effective combined vaccine against *Streptococcus* sp. and *Vibrio anguillarum* has also been developed (Sakai et al. 1995).

CHAPTER 5 FISH IMMUNOLOGY

5.1. Fish Immune System

Fish are a heterogeneous group of animals that are physiologically and anatomically very different. They are divided in the classes Agnatha, Chondrichthyes, and Osteichthyes. Agnatha (Cyclostomes: hagfish and lamprey) lack a well-structured mouth with bones such as maxillary and palatine, and has a cartilaginous skeleton. Chondrichthyes (Elasmobranches: shark, rays) do not have a swim bladder and ossified bones, but only a cartilaginous structure. Osteichthyes (salmon, tilapia) have ossified bones. Teleost are included in the class Osteichthyes. Complexity of the immune system differs substantially among Cyclostomes, Elasmobranches, and Osteichthyes. It is possible to observe a gradual increase in complexity of the immune system (immune organs, cells, soluble factors, and receptors) among these classes.

In mammals, the main lymphoid organs are thymus, spleen, bone marrow, lymph nodes, and gut-associated tissues. Fish have lymph vessels, but lymph nodes have not been observed, at least not with the structure present in mammals. Bone marrow and germinal centers also do not exist in fish, so other organs and tissues perform their functions (Zapata et al. 1992; 1996). The main lymphoid organs in teleost fish are thymus, kidney, spleen, and gut-associated lymphoid tissue. In Elasmobranches the main lymphoid organs are thymus, Leydig's organ, spleen, gut-lymphoid aggregates, intestinal spiral valve, and epigonal organs. However, there is variability in the importance and lymphoid function of these organs, among the different genera of cartilaginous fish

(Culbreath et al. 1991; Rumfelt et al. 2002). Lampreys do not have true lymphoid organs, but lymphohemopoietic tissues are present in various locations such as within the nephros and intestinal lamina propria. Neither lampreys nor hagfish have a thymus, but lymphoid accumulations that could be homologous to thymus, are present in the branchial area (Mattison and Fange, 1977; Rowley et al. 1988). In fish, as in mammals, there is an involution of the thymus with age; and in fish this process can also be related to season or hormonal changes associated with sexual maturation (Honma and Tamura, 1984). In some fish such as rays, flatfish and cod, the thymus continues to grow after sexual maturation, whereas in eels it has already involuted before sexual maturity. In carp and primitive sharks, the thymus does not appear to involute. In *Zoarces viviparus* (Bly, 1985) the thymus reaches its maximum size at 1 month preparturition when it occupies 0.83% of the total body volume. Thereafter, it decreased in size and by 3 months postparturition occupies only 0.048% of the total body volume. Thymus involution is characterized histologically by an increase in connective tissue, a decrease in lymphocyte density, infiltration of adipocytes, the presence of reticular cells and collagenous fibers, and the formation of epithelial cysts. However, the relationship of such changes to the immune capabilities in older fish is not clear.

In mammals, the outer layer of the skin (stratum lucidum) is keratinized and confers a physical barrier to the penetration of pathogens. In fish this layer is not keratinized but is covered with mucus. The mucus protects the skin, decreasing the ability of pathogens to attach to the skin, and it also contains several antibacterial compounds, such as lysozyme and immunoglobulin.

The fish immune system includes cells and soluble mediators that are involved in the innate and acquired immunity. Fish present both B and T-cells that are involved in acquired immunity. Although T-cells have not been formally identified in fish because they lack specific molecules such as CD4, CD8 or CD3, the genes encoding polypeptides homologous to the TCR molecules have been reported in teleosts and elasmobranchs (Partula et al. 1995). Although in mammals the T-cells develop only in the thymus, it appears that in fish an extrathymic population of T-cells is present. The results of some studies have suggested the possibility that the lymphoid cell populations of the thymus and kidney are acquired independently, as it has been observed in early thymectomy fish that still have a normal lymphocyte population in the kidney (Grace and Manning, 1980). B-cell lymphocytes can be recognized in fish by the presence of immunoglobulins on the cell membrane.

Macrophages, polymorphonuclear cells and non-specific cytotoxic cells are involved in innate immunity in fish, as in mammals. Non-specific cytotoxic cells (NCC) in fish are considered to be equivalent functionally to mammalian natural killer cells (NK). NCC can lyse a wide spectrum of mammalian tumor cells and protozoan parasites (Evans and Jaso-Friedmann, 1992) through the mediation of function-associated molecules on the cell membrane (Harris et al. 1992). NCC cells are the smallest leukocytes possessing a very pleomorphic, clefted nucleus and relatively little cytoplasm with no cytoplasmic granules. Villi are present on their surface, and they attach to their target using long, membranous filaments, allowing many binding sites (Secombes, 1996). Unlike mammal NK cells, these cells do not contain cytoplasmic granules. For this reason granule exocytosis is unlikely to be a major factor in their killing action (Evans

and Jaso-Friedmann, 1992). In hagfish only a single population of granulocytes has been reported (Mattison and Fange, 1977), but different subpopulations are distinguishable in lampreys (Rowley et al. 1988). In Elasmobranches several subpopulations of granulocytes have been identified, but there is an enormous heterogeneity among species (Rowley et al. 1988). In teleost fish, different types of polymorphonuclear cells have been detected, but there is an enormous variation among species. Furthermore, eosinophilic and basophilic cells, when present, are not equivalent in function to those corresponding to mammals (Rowley et al. 1988; Zapata et al. 1996). In fish, as in mammals, macrophages act as antigen presenting cells through MHC II, they activate lymphocytes releasing IL-1, and they release reactive oxygen and nitrogen molecules for killing pathogens (Ellsaesser and Clem, 1994). Fc receptors have also been identified on fish macrophages (Haynes *et al.*, 1988).

In Elasmobranches and Osteichthyes, as in mammals, antibodies represent the humoral section of the adaptive branch of the immune system, but differences are present. In Cyclostomes immunoglobulins have been observed in hagfish (Kronenberg et al. 1994), but not in lamprey (Varner et al. 1991). IgM is present in fish, and some species also produce IgG or unique Igs, but IgA, IgD, IgE have not been found. In cartilaginous fish, at least sharks, skates and rays, three different types of immunoglobulins have been found: IgM, IgNAR and IgW (Rumfelt et al. 2002; Voss and Sigel, 1972). IgM exists in approximately equal amounts as a monomer (7S) and a pentamer (19S). In teleost Igs appears to be a tetramer composed of four monomeric subunits, each containing two heavy or larger (~72 kDa) protein chains and two light or smaller chains (~27 kDa). In most species of fish studied, no different immunoglobulin isotypes have

been observed, but this could be caused from a lack of detection (Lobb and Olson, 1988). In fish the assembly of antibody molecules via disulfide crosslinking appears to be a means by which structural diversity is generated. This is distinctly different from what is observed in mammalian species where intersubunit disulfide cross-linking appears to be employed in a more uniform manner (Sanchez and Dominguez, 1991). In mammals, somatic mutations cause a production of antibody with an increased affinity for the antigen. Generally the process of somatic mutation has not been detected in fish, with the exception of some species of elasmobranches (Hinds-Frey et al. 1993). In mammals, a second exposure to an antigen stimulates a greater immune response with a faster and greater production of immunoglobulins (mainly IgG). In contrast, in fish there is a lower production of immunoglobulins after a second exposure to a pathogen compared to the logarithmic increase in monomeric IgG observed in mammals (Arkoosh and Kaattari, 1991). Studies showed that during a secondary response, the increase of specific antibody-producing cells is caused by a clonal proliferation of native and primed lymphocytes, indicating that in fish immune memory is not well developed (Brooks and Feldbush, 1981). Another important difference among the immune system of fish and mammals is the absence of IgA in fish, even if a homologous function of IgA could be present. Some studies have detected antigen specific antibody induced in the mucus of the skin in orally vaccinated fish, while little serum antibody was produced (Rombout et al. 1993). This is similar to what happens in humans, where the local exposure to a pathogen on a mucosa stimulates IgA production in all the body mucosa.

Fish have humoral components of the innate branch of the immune system. Components of the classic and alternative pathways of the complement system have been

identified in fish (Culbreath et al. 1991; Yano, 1996). In general, the complement system in fish is effective against non-virulent gram-negative bacteria, but not against virulent gram-negative and gram-positive bacteria. The alternative complement pathway can be inhibited by the large amount of sialic acid present on virulent strains of gram-negative bacteria (Iida and Wakabayashi, 1993). In Cyclostomes, only components of the alternative complement pathway have been detected, but the complement system lacks a cytolytic activity (Ishiguro et al. 1992; Nonaka, 1994). Elasmobranches possess the classical and alternative complement pathways that stimulate the formation of the membrane attack complexes on target cells (Culbreath et al. 1991). The complement system pathways in bony fish are very similar to those of mammals (Yano, 1996).

Lysozyme is important for fighting all types of pathogens. Lysozyme is distributed mainly in tissues rich in leucocytes, such as the head kidney, skin, gills, and the alimentary tract. Fish lysozyme, in contrast to mammalian, has an antibacterial activity against not only against gram-positive bacteria but also gram-negative bacteria in the absence of complement (Grinde, 1989). Interferon (IFN) has been detected in bony fish, but not in cyclostomes or cartilaginous fish (Yano, 1996). Transferrin and C-reactive proteins have been found in fish with a similar activity as in mammals (Nakanishi et al. 1991; Yano, 1996).

Cytokines, TCR components, major histocompatibility complex I and II (MHC I and MHC II) receptors have been identified in Elasmobranches and Osteichthyes, but the genetic polymorphism of MHC genes is considerably lower than in mammals (Knight et al. 1998; Secombes et al. 2001; Zapata et al. 1992). A few cytokines, but no MHC molecules, have been isolated from Cyclostomes (Kronenberg et al. 1994).

One of the major differences between the immune system of fish and mammals is the effect that temperature has on the system. In fish, the rate of the development of the immune system is influenced more by the external temperature and by the size of the fish than by age (Tatner, 1996). The higher the temperature, the faster the fish grows and the earlier the immune system develops. Rainbow trout start to produce antibodies when they reach a weight of approximately 0.24 g without any dependence on their age (Tatner and Manning, 1983; Tatner, 1986).

Seasonal changes in water temperature influence the functions of the fish immune system. For example, the number of peripheral blood lymphocytes is minimum in winter and maximum in the summer, although there are exceptions as in the brown trout where the levels of lymphocytes are low in winter and summer and high in spring and autumn. In winter, it is possible to observe regression in lymphoid tissues, particularly the thymus. In spring and summer there are waves of thymocyte production, while degenerated lymphoid cells can be found from the beginning of autumn and throughout winter. Higher temperatures cause faster and higher antibody production (Rijkers et al. 1980). Temperature has a different influence on the various parts and cells of the immune system. For example, virgin T-cells rather than memory T-cells, B-cells or accessory cells are particularly susceptible to the inhibitory influences of low temperature (Bly and Clem, 1992; Miller and Clem, 1984). Low temperatures have a higher inhibitory effect on T-cells than on B-cells. A possible explanation for this phenomenon is that B-cells have a higher capability to modify the fluidity of their plasma membrane. B-cells can rapidly desaturate stearic acid to oleic acid whereas T-cells can not. Moreover, T-cells accumulate relatively large amounts of stearic acid in membrane-associated

phospholipids causing an increase of membrane viscosity. This may explain the suppressed function at low temperatures. In fact, a lower membrane viscosity would affect receptors and signal pathways that involve membrane-associated proteins (Bly et al. 1990). Even if low temperatures have an inhibitory effect, the fish immune system is able, at least in part, to counterbalance this effect. For example, in channel catfish serum complement lytic activity is low in winter, while the antibody affinity increases. In this way antibody can act as an opsin and activate the complement cascade (Clem and Small, 1970; Hayman et al. 1992). A similar mechanism involves C-reactive proteins in channel catfish. CRP levels are low in winter, but at the same time their ligand avidity increases. Temperature also influences the mammalian immune system, but in a minor way. This difference is caused mainly by the ability of mammals to maintain a constant body temperature.

The immune system of sharks (*Epalzeorhynchus* sp.) has not been studied in any research. However, because sharks belong to the family Cyprinidae, it is very plausible that there are similarities between the shark, the common carp, *Cyprinus carpio*, and the danio, *Danio rerio*, immune systems and ontogenesis of the immune systems. Because sharks are warm water fish, as danio, it is plausible the timelines of the ontogenesis of the immune system is more similar between these two species than between shark and carp.

5.2. Ontogenesis of the Immune System

The ontogenesis of the fish immune system has been studied only in a few species (Ellis, 1988; Honma and Tamura, 1984; Tatner, 1996), such as nurse shark, *Ginglymostoma cirratum* (Rumfelt et al. 2002), channel catfish, *Ictalurus punctatus* (Petrie-Hanson and Ainsworth, 2001), danio, *Danio rerio* (Hansen, 1997; Hansen and

Zapata, 1998; Trede and Zon, 1998; Trede et al. 2001; Willett et al. 1997ab; Willett et al. 1999), common carp, *Cyprinus carpio* (Botham and Manning, 1981), rainbow trout, *Salmo gairdneri* (Grace and Manning, 1980; Tatner and Manning, 1983; Tatner, 1986), viviparous blenny, *Zoarces viviparous* (Bly, 1984; 1985), European flounder, *Platichthys flesus* (Pulsford et al. 1994), channel catfish, *Ictalurus punctatus* (Petrie-Hanson and Ainsworth, 2001), and angler fish, *Lophius piscatorius* (Fange and Pulsford, 1985).

Histological, immunological and biomolecular techniques have been used in these studies. In histological analyses, tissues are first stained and then observed microscopically. Immunological techniques utilize rabbit or mouse monoclonal antibodies against the putative B and T-cells that can be detected by ELISA or fluorescent staining techniques. Biomolecular techniques utilize the expression of several genes involved in the ontogenesis process. One of the most delicate steps in this type of analysis is the design of specific mRNA primers. Usually mRNA is isolated, amplified by PCR and identified by Northern Blot analysis. In situ hybridization techniques can be used for the detection of the time and of the organs where specific genes are expressed.

5.3. Immune Organs and T-Cell Ontogenesis: Histological Approaches

The thymus is the first immune organ to develop in fish and the first to become lymphoid (Bly, 1985; Botham and Manning, 1981; Grace and Manning, 1980; Tatner and Manning, 1983; Tatner, 1986; Trede et al. 2001; Willett et al. 1999). The second organ to develop is the kidney followed by the spleen. The gut-associated lymphoid tissue becomes lymphoid later than the kidney and the spleen. In general, the spleen remains predominantly erythroid throughout the life with relatively few lymphocytes. Tatner and Manning (1983) monitored the growth and the cellular composition of the lymphoid

organs in rainbow trout from 1 to 15 months of age. Thymus, kidney and spleen grew steadily over this period, but all attained their maximum relative weights expressed as a percentage of body weight, at 2-3 months of age, indicating a time of intense activity and importance in the maturation of the lymphoid system. The total number of leukocytes in the organs increased with age, but when the size of the organ and the size of the fish were taken into account, the actual leukocyte numbers decreased with age. The thymus showed intense mitotic activity in the first few months, then slowed down. There were no signs of massive cell death, and it was assumed that emigration of cells to the peripheral lymphoid organs must have been occurring. Histological changes indicative of involution were present in the thymus from 9 months onward. The spleen and the kidney showed an increase in melanin deposition with age, with the kidney retaining a more varied range of leukocyte types than the spleen. The number of circulating lymphocytes in the blood remained remarkably constant throughout the first year of life.

The progressive development of the immune organs was also observed in common carp (van Loon et al. 1982). The thymus contained the largest lymphoid cell pool, 70% or 3×10^5 cells at day 28, in the first 4 weeks. At two months of age the total lymphocyte cell pool, 5×10^6 cells, was distributed for 38% in the mesonephros, 32% in the thymus, 15% in the pronephros, 12% in the peripheral blood and 3% in the spleen.

In *Zoarces viviparus*, a viviparous marine fish, thymus has a paired structure localized behind the last gill arch (Bly, 1985). The thymus lies in the angle between the operculum and the body wall, and is covered by a single layer of epithelium. It is evident in the embryo from 3 months preparturition, and at two months preparturition, two zones with different lymphocyte density can be observed. The first is localized on the side, is

densely stained, and is comprised of mainly lymphocytes and few reticular cells. The other inner zone is lightly stained and contains fewer lymphocytes. However, there is no distinct boundary between the two zones. The thymus is covered by a thin connective tissue capsule and by collagen fibers. Macrophages can also be found. The head kidney is seen at 3 months preparturition, and it contains mainly a few kidney tubules surrounded by small erythrocytes. At 1 month preparturition the kidney also contains lymphoid tissue, where granulocytes and macrophages can be found. The spleen is first observed at two months preparturition. The organ is in close contact with the pancreas.

Differentiation in the spleen is evident at two months postparturition when two zones are apparent: lightly stained lymphoid areas aggregated around the trabeculae, and dark staining areas composed mainly of erythrocytes. These two zones may be analogous to the white and red pulp of mammalian spleen. In the spleen, granulocytes and macrophages are also present.

In rainbow trout (Grace and Manning, 1980; Razquin et al. 1990; Tatner and Manning, 1983; Tatner, 1986), thymus is first observed at 4 days before hatching. At this stage it consists of a thickened area of the pharyngeal epithelium containing a few lymphoid cells interspersed among the epithelial cells, and of a thin connective tissue. At 4 days after hatching, thymic stellate cells, numerous thymocytes, and some macrophages are present. The first connective tissue trabeculae in the thymic parenchyma are observed about 14-21 days after hatching, and incipient subcapsular and outer thymic zones appear at 1 month age. Thymic cysts develop from 1-2 months after hatching. Renal lympho-haemopoietic tissue is observed at 4 days before hatching, and is formed by discrete foci of erythroid cells located among the reticular cells and blood sinuses of the

intertubular loose connective tissue of the developing kidney. At hatching, those foci tend to fuse and contain myelocytic cells interspersed among the cell processes of reticular cells. Scattered large lymphoid cells can be clearly observed at 4 days after hatching, and from this stage the amount of lympho-haemopoietic tissue increases in the kidney, retaining a similar structure in both pro- and mesonephros, except for the absence of renal tubules in the former. The spleen develops slowly, containing a poorly organized haemopoietic red pulp at 12 days after hatching, formed by loose cell cords, mainly occupied by erythroid cells and enlarged blood sinusoids. Some scattered lymphocyte, macrophages and melanomacrophages occurred in the spleen about 14 days after hatching. Small amounts of splenic white pulp can be observed in 2 month old trout, when ellipsoids also develop.

The thymus of the channel catfish, *Ictalurus punctatus* (Petrie-Hanson and Ainsworth, 2001), is first colonized by monocytes, suggesting that these cells may help thymocyte proliferation and differentiation. The first immature lymphocytes can be seen in the thymus after 5 days. Mature lymphocytes are detectable at 10 days post-hatch. The intermediate stage of T lymphocytes is concentrated in the peripheral lighter staining area (cortical zone); meanwhile mature lymphocytes are concentrated in the central darker staining area (medulla region). Until approximately 3 months post hatch, the total number of lymphocytes increases, then reaches a plateau.

In zebrafish, *Danio rerio*, a thymic primordium consisting of two layers of epithelial cells appears at 54 h after fertilization (Willett et al. 1997a). By 65 h post-fertilization it is colonized by immature lymphocytes. Later, lymphocytes and epithelial cells differentiate for the next 3 weeks, but at 1 week the thymus begins to be

morphological mature. At 72 h post-fertilization kidney glomeruli can be seen. At 96 h post-fertilization the pronephros begins to contain hematopoietic cells, mainly developing erythrocytes and granulocytes. Fibroplastic reticular cells, that will constitute the supporting apparatus for the developing hematopoietic cells, appear at 7 days post-fertilization. Lymphocytes are detected after 3 weeks.

The first appearance of lymphocytes within an organ, rather than the first appearance of the organ itself, is considered to be a better index of the maturation of the lymphoid system (Tatner, 1996). The reproductive strategy among fish (such as the length of egg incubation) and the environmental temperature are the main factors that influence the time of maturation of the immune organs. The warmer the water temperature, the faster development occurs. In general, the longer the hatching time, the slower the development. For example trout eggs are incubated at 14°C and hatch around 18 days; however, when they are incubated at lower temperatures, they develop slower. Carp eggs are reared at 22°C and hatch after 36 h. The gestation period in *Z. viviparous* is 4-5 months. Table 5-1 summarizes the first appearance of the immune system organs; Table 5-2 the first appearance of lymphocytes in those organs, and Table 5-3 when the thymus can be considered immunologically mature.

Development of cell-mediated protection can be better analyzed using allograft rejection responses. Allograft rejection in carp can occur as early as day 16 post-hatch (Secombes and van Groningen, 1983), at day 26 post-hatch in rainbow trout, and at 2 months in *Tilapia mossambica*. These results suggest that cytotoxic T-cells mature earlier than B-cells.

It still is not well understood if during ontogenesis lymphocytes migrate from the thymus to the kidney. The results of several studies have outlined the possibility that the lymphoid cell populations of the thymus and kidney are acquired independently. In fact, early thymectomized fish have a normal lymphocyte population in the kidney (Grace and Manning, 1980).

5.4. Immune Organs and T-Cell Ontogenesis: Biomolecular Approaches

Zebrafish have been used as a model in biomolecular studies for immune system ontogenesis (Trede and Zon, 1998; Trede et al. 2001). One main reason is that the genome of the zebrafish has been almost completely cloned. Moreover, because of the small size (5 cm) of this fish, they are reared easily, they can be stocked at high density (up to 70 adult per 19 L tank), their generation time is relatively short (3 months), and females produce a large number of progeny (200-300 every week). The embryos develop outside the mother's body and are easily accessible for physical manipulation, visual inspection under dissecting microscope, whole mount in situ hybridization, and gene transfer experiments. Thanks to the relatively short generation time, zebrafish lineages may be created for specific mutant genes, and may be used for positional cloning of such mutant genes. Zebrafish mutants with severe hematopoietic defects can still live beyond the onset of thymopoiesis, this permits the analysis of lymphocyte development in mutants that would be destined in the mouse to suffer early embryonic death from anemia or other developmental defects (Trede et al. 2001). The juveniles of this fish are almost transparent allowing a direct visualization of the temporal and spatial development of lymphoid system.

The utilization of green fluorescent protein genes, the expression of which is under the control of the promoter of the studied genes (such as *lck*, for example), helps in the direct visualization of the ontogenesis of the immune system. Additionally, proto-oncogenes can be expressed under the control of the studied genes so as to cause tumors. The animals with these malignant tumors can be utilized in several ways. For example, monoclonal antibodies could be produced against zebrafish homologs of T-cell markers such as CD3, CD4 and CD8. These transgenic experiments would also provide an animal model of leukemia in lower vertebrates (Trede and Zon, 1998).

New techniques to study gene expression will apply retroviral insertions for the generation of zebrafish mutants. A major advantage of this approach is the ease with which genes responsible for the mutant phenotypes can be cloned (Trede et al. 2001). These characteristics make the zebrafish an ideal model for studying the ontogenesis of the vertebrate immune system.

As soon as the first lymphocytes are visible they are still not functionally active or mature. For this reason some studies (Hansen, 1997; Hansen and Zapata, 1998; Trede and Zon, 1998; Trede et al. 2001; Willett et al. 1997ab) have analyzed the gene expression of the enzymes including *RAG1*, *RAG2*, *TdT*, *lck*, *GATA*, *ikaros*, or *TdT*, as markers of the maturation of the immune system. These enzymes are involved in the maturation process of the T and B-cells acting on the TCR and Igs genes rearrangement. These studies have confirmed the results obtained by histological investigations (Willett et al. 1997a). Brain and thymus express *ikaros*, indicating where early lymphoid development occurs. Afterward, the thymus also expresses *GATA3*, *RAG1* and *lck* indicating the localization of T-cell development (Trede et al. 2001). In other studies (Willett et al. 1997b; Trede and

Zon, 1998) expression of *RAG1* and *RAG2* were first observed in the thymus at 92 h post-fertilization marking, perhaps, the first rearrangement of TCR genes. At this time the thymus is heavily populated with lymphocytes. The thymus enlarges for at least 1 month continuing to express the *RAG* genes and changing shape from spherical to crescent. No distinction of medulla and cortex is present at this stage. The expression of the gene *lck* is temporally and spatially parallel to the expression of the two *RAG* genes. *Ikaros*'s mRNA is found for the first time in the Intermediate Cell Mass (an intraembryonic region between the somites and the yolk sac) at 24 h after fertilization. At day 2, *Ikaros*'s mRNA is expressed in the dorsal aorta; and at day 3, it is expressed in the thymus. Pronephros and kidney, which are respectively the larval and adult sites of hematopoiesis, do not express *RAG1* until day 21 indicating the later lymphoid function development of these organs (Trede et al. 2001). The DNA polymerase Terminal Deoxynucleotidyl Transferase (*TdT*) is one of the enzymes involved in the generation of functional diversity during immunoglobulin and T-cell rearrangement. Northern blot analysis shows that *TdT* mRNA is highly expressed within the thymus and to a lesser extent in the pronephros (Hansen, 1997). This research has also showed that *RAG1* and *TdT* genes are expressed also in the intestine, where few lymphocytes are present. This result suggests that the intestine of fish, as in mammals, may serve as an alternative site for lymphocyte development. That could indicate that the GALT is a location for gamma-delta T-cell homologues.

5.5. B-Cell Ontogenesis

The kidney seems to be the first lymphoid organ where IgM bearing cells can be found during the ontogenesis of the immune system (Razquin et al. 1990; Secombes and van Groningen 1983). Detection of IgM on the cell surface is a technique utilized to

determine the age of maturation of B-cells. In common carp immunoglobulins were detected on the surface of cells in thymus and pronephros from 14 days after fertilization at 21°C, and in mesonephros and spleen, from days 28 (Koumas-van Diepen et al. 1994). From the same research, it was observed that the age of fish and the percentage of surface immunoglobulin (sIg) cells in the lymphoid organs were positively correlated, with the lowest percentage in 2 week old fish and the highest in the oldest fish. The percentage of sIg cells increased gradually from 3 to 16 months of age, with values being respectively 15.8-48.1% for blood, 9.7-21.6% for pronephros, 7.2-16.8% for mesonephros, 15.9-21.6% for spleen and 1.5-3.7% for thymus. The percentages of cytoplasmic Ig cells were very low or absent in spleen, thymus and blood. Plasma cells were not found in the kidney until the fish were 1 month old (0.17%), and increased to 1% of the total cell population by 8 months. This was supported by a steady increase in serum Igs from 3 weeks onward. In *S. salar* membrane IgM was detected at 41 days post-hatch, this coincided with the onset of first feeding (Ellis, 1977). From this stage, the percentage of positive lymphocytes increased steadily reaching 80% by day 48 post-hatch.

In rainbow trout cytoplasmic IgM was first observed in embryos at 12 days before hatch with eggs reared at 14°C (Castillo et al. 1993; Razquin et al. 1990). At this stage no cells with sIgM were present. Lymphocytes with sIgM were first observed at 8 days before hatch. Unfertilized eggs contained detectable amounts of IgM indicating that transfer of IgM from mother to embryo is possible in salmonids. Cells with sIgM first appear in the kidney 4-5 days after hatching. By 1 month after hatching, IgM positive cells also appeared in the spleen and thymus. Enzyme-histochemical demonstration of the alkaline and acid phosphatase and non-specific naphthyl acetate esterase enzymatic

activities in the non-lymphoid cells indicated that a certain degree of maturation of the cellular stroma of the developing lymphoid organs of trout was reached before or at the time when IgM-expressing cells could be observed. This could suggest the possibility that both macrophages and some stromal cells are involved in the maturation of B-cells. However, reticular cells with alkaline phosphatase are not observed in the spleen red pulp of trout. This could explain the difference in haemopoietic and lymphoid functions between kidney and spleen. In splenic parenchyma, reticular cells with acid phosphatase activity are present before the appearance of the first IgM positive cells. That cell type appears to form the extravascular microenvironment of the red pulp, which is an important erythropoietic site (Zapata, 1983). In contrast, the development of alkaline phosphatase reticular cells in the white pulp of the spleen is not related to the occurrence of the first IgM positive cells in this organ. The development of IgM positive cells in the thymus might be related to the development of the connective tissue trabeculae, rather than with the differentiation of alkaline phosphatase and acid phosphatase-positive cells. In fact, cells with IgM are not present in the thymus until the development of thymic trabeculae, and IgM positive cells are concentrated at the border between the thymus inner and outer zones where trabeculae ramify into several branches.

In mammals, macrophages play an important role in the B-cell maturation process. It appears that in fish these cells have the same function. Melanomacrophage centers may be functionally analogous to the germinal centers of higher vertebrates where the localization of antigen on the surface of dendritic cells has been implicated in the production of B memory cells, feedback inhibition of antibody production, switch from 19S to 7S immunoglobulin production, and increased affinity for the antigen (Razquin et

al. 1990). Furthermore, kidney and spleen of trout fry are functional phagocytic organs prior the acquisition of lymphoid cells (Tatner and Manning, 1983). Development of splenic ellipsoids, which are considered involved in antigen trapping in fish, appear to be related in time to the differentiation of the white pulp rather than with the early differentiation of splenic IgM positive cells (Lamers and De Hass, 1985).

Similar results have been observed in channel catfish (Petrie-Hanson and Ainsworth, 2001). In renal interstitial tissue, macrophages are present before B-cells suggesting a possible role in B-cell differentiation. In channel catfish larvae mature B-cells appear in the anterior kidney before their detection in any other organ, confirming the role of the kidney in early lymphopoiesis. B-cells are detected in the kidney at 7 days post-hatch, in the thymus at 10 days, and in the spleen at 14 days after hatching. Interestingly, in research conducted by Petrie-Hanson and Ainsworth (2001) Igs positive cells were absent in the thymus from day 14 to 19. On day 21 putative B-cells were observed again in the thymus. This suggests a wave-like, B-lymphocyte maturation process in the thymus. At hatch, the spleen of catfish contains only erythrocytes and blast cells. Monocytes are present by day 3. By day 5 neutrophil and immature B-lymphocytes are present. Mature B-cells are observed from day 14 after hatching. At this time there is still not any recognizable organization of the splenic lymphoid compartment. T-cells can be found by day 28. There is an increase in the T-cell population until 2 months post-hatch and at this stage T-cells appear to be more numerous than B-cells. T and B-lymphocyte populations are localized adjacent to the arterioles and along some of the larger veins. B-lymphocytes are also present in the outer edge of the lymphocyte area.

The response to thymus-dependent and thymus-independent antigens in fish involves different immune cells, and these responses develop at different ages. B-cells respond to thymus-independent antigens, while helper T-cells and accessory cells respond to thymus-dependent antigens. Antibody production has been observed in 23 days old rainbow trout, however, juveniles respond earlier to thymus-independent than to thymus-dependent antigens (Paterson and Fryer, 1974). Carp fry, 4- weeks old, were unable to mount a plaque-forming cell response to sheep red blood cells, that are thymus-dependent antigens (van Loon et al. 1982). In contrast, 8-week-old carp fry were able to mount a primary humoral response against human gamma globulin, a thymusdependent antigen, and against formalin-killed *Aeromonas salmonicida*, which is a thymus-independent antigen. Rainbow trout also mount a response to thymus-independent antigens earlier (2 months old age) than to thymus-dependent ones (3 months old age) (Tatner, 1986). If juveniles are exposed too early to thymus-dependent antigen, they can develop a tolerance state. These results are important for vaccination strategies. Fry can be successfully vaccinated only when they can respond to antigen, and vaccines based on thymus-independent antigens can be used earlier.

In Elasmobranches, Leydig's organ, the intestinal spiral valve, spleen and the epigonal organs are important in B-cell maturation (Rumfelt et al. 2002). In Elasmobranchs the epigonal and the Leydig organs are homologous to the mammalian bone marrow. The Leydig organ is attached to the esophagus and consists of a reticulum packed with large number of leukocytes with few arteries and capillaries present. The leukocytes, including neutrophil, eosinophil, and other granulocytes, are less mature in the Leydig organ than in the blood, so this tissue seems to be important for myeloid cell

development. Putative lymphocytes of various sizes are abundant in this organ and they form loose follicle-like aggregates with scattered plasma cells. The epigonal organ is similar in structure and organization, and it is physically attached to the gonads. It is a primary lymphoid tissue for the entire life of the animal. At least one of these organs is present in all cartilaginous fish; for example in nurse shark only the epigonal organ is present. As in other vertebrates, the spleen is highly differentiated, has a vascular structure, and is the major site for antigen presentation for antibody production.

In the cartilaginous sharks, skates, and rays, three different types of immunoglobulins have been found: IgM, IgNAR and IgW (Rumfelt et al. 2002). IgM exists in approximately equal amounts as a monomer (7S) and a pentamer (19S). In neonatal nurse sharks there are very low levels of IgM; at 1 month of age, the level of IgM is similar to the adult one. However, some IgM may be passed from the mother by the egg yolk. In neonatal sharks, high levels of a different IgM are present in the blood. Cells of the spleen and epigonal organs secrete other forms of IgM, and a germline-joined non-diverse VDJ gene encodes it. This type of IgM is secreted throughout the adult life only in the epigonal organ. IgM is first expressed in the liver, then in the kidney, spleen, and Leydig/epigonal organ when the juveniles are close to hatch. IgNAR is expressed mainly in adults, while IgW has been identified only in the supernatant of in vitro cultures of splenocytes. It is also interesting to note that in adult sharks MHC class II mRNA is highly expressed in the spleen, thymus, gill, and intestine (Ohta and Flajnik, unpublished results). In the spleen, MHC II is expressed on sIgM⁺ cells (B-cells) and on large IgM⁻ and IgNAR⁻ cells (dendritic-like cells). The adult spleen is organized in three zones: (1) small circular white pulp areas consisting entirely of cells expressing IgM on

the surface, (2) large white pulp areas presenting cells without IgM and with few scattered IgM and IgNAR secretory B-cells, (3) red pulp with IgM putative secretory cells localized principally close to venous sinuses. The $Ig\gamma^-$ and MHC II $^-$ cells in the second zone are associated with MHC II $^+$ cell (dendritic cells) and they resemble lymphocytes in size and appearance. For these reasons they are considered putative T-cells. In newborn sharks, the spleen is populated mainly by sIgM $^+$ cells (B-cells), and these cells have low expression of MHC II. They are also present in lower numbers than in adults. T-cell zones are not yet present. In newborn shark the epigonal organ contains developing granulocytes, blast cells, lymphocytes and plasma cells. The genes RAG1 and TdT are expressed in these organs during the entire life of the animal, and the transmembrane form of IgM mRNA is predominantly found. These data and the fact that the present sIgM cells are not secretory, suggest that in reality these cells are more likely developing naïve B-cells. The expression of RAG1 gene is 10 times higher in the thymus than in the epigonal organ, and this gene is not expressed in spleen or muscle. This confirms that the thymus is the site for T-cell maturation in shark (Rumfelt et al. 2002).

Data collected in several studies (Irwin and Kaatari, 1986; Kaatari and Irwin, 1985; Sanchez et al. 1995) have shown that there is an organ dependent heterogeneity in B-cell populations. These differences are also related with the specific function of the immune organs. The anterior kidney of coho salmon possesses lymphocytes with a more restricted antigen recognition profile of antibody specificities than do lymphocytes from the posterior kidney or spleen. The heterogeneity profiles of the posterior kidney resemble more closely those of the spleen than those of the anterior kidney. The precise causes of the difference in heterogeneity are not known. Such differences could be caused

by variation in antibody affinity or to the actual absence of select antigenic specificities in different B-cell populations. Differences in the response to mitogens can be also observed among B-cell populations of different organs. Anterior kidney cells can respond only to LPS, while splenic cells can respond to LPS and PPD. These results must be related to the function of these organs: the anterior kidney is more a hemopoietic center and can be considered the homologue of mammalian bone marrow.

Differences in organ function can be also observed in the antigen trapping process. In the spleen antigens are probably held as immune complexes, and are located extracellularly, associated with the metalophil reticular fibers present mainly in the ellipsoid walls, scattered throughout the peri-ellipsoid areas, and the melanomacrophage centers. In the kidney, tissue antigens are present mainly intracellularly within the reticulo-endothelial system and free macrophages. The thymic and the reticulo-endothelial system of the heart atrium play no part in the uptake of antigen. The antigen trapping process could be involved in the induction of B memory cells and in the negative feedback control of antibody production (Ellis, 1988).

5.6. Nonspecific Immunity

Non-specific immunity involving both cellular and soluble factors plays an important role in the defense mechanisms of fish both in the adult and during earlier stages of development. Fish are most likely entirely dependent on non-specific immunity during the period of free-living existence prior to immune system maturation. Injection of colloidal carbon particles has been used to study the phagocytic activity in different tissues (Johnson et al. 1982; Zapata et al. 1997). Four day old trout still do not have a lymphocyte population. Carbon particles are phagocytated mostly by free macrophages

present in connective tissues, under the skin, in the gut, and in the gills. Some macrophages are also observed in the kidney. By day 18 the kidney has become efficient at trapping carbon. At 8 months the main sites of localization of phagocytosed material are the spleen, kidney, heart, and macrophages in the blood. In 2 week old carp, the phagocytic pattern is similar to that in the adult, and the kidney and spleen trap the largest amount of carbon.

In addition to the nonspecific lectins and hemagglutinins found in fish eggs and newly hatched fry, fry possess an efficient phagocytic system (Tatner and Manning, 1983; Tatner and Horne, 1984). Rainbow trout fry 4 days post-hatch have an efficient phagocytic system, and administered carbon is engulfed by free-wandering macrophages that accumulate under the skin and in the connective tissue, gut, and gills. By day 14 post-hatch the pattern of antigen-trapping was similar to that found in adults, with the fixed macrophages of the spleen and kidney sequestering the carbon particles from the blood stream.

Passive immunity from mother to fry has been demonstrated in a few species, and C-reactive protein and lectin-like agglutins, that should be maternally derived, have been found in ova of several fish (Tatner, 1996). In rainbow trout the levels of IgM in the whole fish increased gradually to reach a peak, in terms of milligrams per gram tissue, at time of hatch, and then slowly declined to initial values by 2 months post hatch (Castillo et al. 1993). Differences in the feto-maternal and neonate-maternal relationships were observed among oviparous plaice, *Pleuronectes platessa*, the viviparous blenny with ovarian gestation, *Zoarcetes viviparous*, and the viviparous swordtail with follicular gestation, *Xiphophorus helleri* (Bly, 1984). The study showed uptake of immunoglobulin

into plaice eggs, the young blenny, and swordtail. The earlier lymphoid development in the blenny compared to the oviparous species could result in the embryo being immunologically competent while still within the ovary, and capable of mounting an immune response against both maternal and sibling antigens. The finding that immunoglobulin can be detected in the eggs and larvae of fish raised the possibility that hen (mother) fish could be vaccinated to provide their offspring with protection against various pathogens. This would be particularly beneficial in the case of pathogens that are transmitted vertically and to which the young are particularly susceptible, such as IPN virus.

5.7. Interactions between the Immune and Neuroendocrine Systems

Neuroimmunoendocrinology is a new interdisciplinary field of research that has recently emerged, and it involves the study of the bidirectional communication between the immune and the neuroendocrine systems. These systems are closely and finely interconnected through innervations and a bidirectional communication that uses the same set of signal molecules, such as hormones and lymphokines. Most of the studies in the neuroimmunoendocrinology field have been conducted on mammals, but fishes have also been target of study, as several recent reviews have shown (Harris and Bird, 2000; Weyts et al. 1999; Yada and Nakanishi, 2002). There are several lines of evidence that the sympathetic nervous system (SNS) plays a role in immune regulation: (1) primary and secondary lymphoid organs are innervated by sympathetic noradrenergic nerves; (2) synapse-like contacts among sympathetic nerve terminals and T and B-cells in the spleen have been observed (Felten and Olschowka, 1987); (3) the vast majority of lymphoid cells express adrenergic receptors, with the exception of Th2 cells (Rammer-

Quinn et al. 1997); (4) norepinephrine (NE) is released in lymphoid organs following immunization (Kohm et al. 2000); (5) NE can alter lymphoid cell functions (Moynihan et al. 2004); (6) the nervous system acts as an endocrine system when activated neurones produce an electric stimulation of the terminal part of axon, thus releasing neuromediators, such as norepinephrine, that will influence the functions of the target cells. In addition, the histological structure of some endocrine glands resembles the structure of some parts of the nervous system, as can be observed in the neurohypophysis, composed mainly of axons originating in the hypothalamus. Also in fish, the sympathetic nerves have a regulatory function on the immune system (Flory, 1989).

External and internal stimuli are registered by the nervous system and passed to the hypothalamus. The hypothalamus, through nerve axons or hormones, inhibits or stimulates the pituitary to release a second group of hormones that will target specific organs, tissues, and glands regulating their physiology. During these processes several negative feedback mechanisms are present to finely control the release of hormones and their effects. For example, under specific stimuli, the nervous system stimulates the hypothalamus to release the thyrotropin-releasing hormone (TRH) that will stimulate the hypophysis to secrete thyroid-stimulating hormone (TSH). TSH will stimulate the thyroid to synthesize and release the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4). Both these hormones will target specific tissues and organs to stimulate precise physiological processes, but they will also produce a negative feedback on both hypothalamus and hypophysis inhibiting the release of TRH and TSH. In this way an excessive production and release of T_3 and T_4 will be avoided.

As mentioned previously, hormones can influence and be released by the immune system. It is well known that hormones released under stress conditions can affect the immune system (Blalock, 1989; Sanders et al. 1997; Schreck et al. 2001), but they are not the only ones. For example, TRH stimulates both the adenohypophysis and T-cells to release TSH (Kruder and Blalock, 1986). TSH, produced by the hypophysis or T-cells, stimulates the thyroid to synthesize and release the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4). Chorionic gonadotropin (CG) can be also produced by T-cells under gonadotropin releasing hormone (GnRH) stimulation by the hypothalamus. Somatostatin can block CG production both from the adenohypophysis and T-cells. The adrenocorticotrophic hormone (ACTH) can suppress the immune response to both T-dependent and T-independent antigens, lymphokines and IFN-gamma production by T-cells, and Igs synthesis and macrophage activation by IFN-gamma (Blalock, 1989). The effect of opioids on the immune system is very variable dependently on the type of opioid peptide and immune cell. For example, alpha-endorphins are potent suppressors of antibody production, but not beta or gamma-endorphins (Johnson et al. 1982). Alpha-endorphins have multiple effects on the immune system dependently on the cell type: they block T helper cells from producing and/or secreting interleukins needed for antibody production, they inhibit B-cells from producing antibody and transforming plasma cells. Beta-endorphins enhance T-cell proliferation and differentiation to cytotoxic T-cells, stimulate cytotoxicity in NK cells, and they are chemotactic factors for monocytes and neutrophils. Neuropeptide Y (NPY) can have a stimulatory or inhibitory effect on the immune system depending of the function and cell type. For example, NPY can influence T-helper cell differentiation; inhibit NK cell activation, release of oxygen

radicals in peritoneal monocytes, and macrophage phagocytic activity; increases monocytes mobilization, leukocytes adherence capability, and immune cell redistribution. Neuropeptide Y can produce a shift in Th2 response by suppressing Th1 cells release of IFN-gamma and stimulating the production of IL-4 by Th2 cells, thus causing an increase in serum antibodies. Neuropeptide Y can also influence the immunomodulatory effects of other neurotransmitters, thereby acting as a neuroimmune co-transmitter (Ahmed et al. 2001; Bedoui et al. 2003).

Gastrointestinal hormones can also modulate the immune system. Substance P increases T-cell proliferation, degranulation of basophils and mast cells, enhances macrophage phagocytosis, stimulates O_2 , H_2O_2 and tromboxane B_2 production (Goetzi et al. 1985; Payan and Goetzi, 1985). Substance P and somatostatin can also modulate cells involved in delayed-type hypersensitivity reactions and cell-mediated immunity. Somatostatin stimulates release of histamine from mast cells, but suppresses histamine and leukotriene D_4 release from basophils. It also suppresses T-cell proliferation. These two hormones can have similar effects, such as stimulating macrophage phagocytosis, but they can also have opposite effects: substance P can increase thymidine and leucine uptake from T-cells in the presence or absence of mitogens, while somatostatin inhibits this process. The effects of these hormones can be different even on the same cell type located in different tissues. For example, tissue mast cells and basophils in the circulation do not release mediators in response to neuropeptides. Instead, nanomolar to subpicomolar concentrations of somatostatin can suppress Ig-E mediated histamine and leukotriene D_4 release (Goetzi et al. 1985). Vasoactive intestinal peptide (VIP) can be produced by mononuclear, leukocytes, mast cells and polymorphonuclear leukocytes

(Blalock, 1989). Vasoactive intestinal peptide can influence the immune system stimulating cytotoxic T-cell, but not B-cell proliferation. This effect can be explained by the presence of VIP receptors on T, but not in B-cells.

The immunoregulatory function of hormones has also been demonstrated in fish. Sex steroids, especially androgens, can suppress the immune system (Hou et al. 1999; Yamaguchi et al. 2001). Melatonin also appears to modulate the immune system (Yada and Nakanishi, 2002). Prolactin and growth hormone enhance both the cellular and humoral immune responses, and are important for the proliferation of B-cells (Sakai et al. 1996a, b, c; Yada et al. 1999). The decrease in immune responses of hypophysectomized fish demonstrated the importance of hormonal modulation of the immune system (Yada et al. 1999; Yada and Azuma, 2002). As in mammals, gastrointestinal hormones, such as substance P, somatostatin, and calcitonin gene-related peptide, may regulate the immune functions of fish (McGillis and Figueiredo, 1996; Ndoeye et al. 1991).

Lymphokines are usually associated with the immune system, but they can also influence the neuroendocrine system. When IL-1 enters the circulation, it can act as a hormonal signal. IL-1 may pass through the fenestrated endothelium in the brain (median eminence) and stimulate corticotrophin-releasing hormone (CRH) secretion from the CRH nerve-terminals that will influence the physiological activity of other parts of the body. In addition, IL-1 may activate the brain endothelial cells to produce IL-1, IL-6, and prostaglandins, and to secrete other mediators into the brain (Tilders et al. 1994). As for hormones, lymphokines can also be produced by the neuroendocrine system, for example astrocytes and glial cells can produce IL-1 (Fontana et al. 1982; 1984). Also cytokines can influence the neuroendocrine systems in fish and regulate the hypothalamus-pituitary

axis in response to infection and inflammation (Cohen and Kinney, 2001; Turnbull and Rivier, 1995; Yada and Nakanishi, 2002).

Hormone and lymphokine receptors have been identified in cells of both immune and neuroendocrine systems. IL-1 has two distinct receptors, Type I and II. Type I is present on T-cells, fibroblasts and endothelial cells; Type II on B-cells and macrophages. IL-1 receptors are present also in the choroid plexus, pituitary gland, and hippocampus.

Even if some hormones and lymphokines are produced and interact with both immune and neuroendocrine systems, some differences are present. For example, ACTH is produced by the hypothalamus, but lymphocytes and macrophages can also synthesize it. The hypothalamus can synthesize two distinct forms of ACTH that differ in their amino acid sequence (1-39; 1-24). Interestingly, both forms of ACTH have the same steroidogenic activity, but only the ACTH 1-39 can regulate the immune system (Johnson et al. 1982). At least in this context, cells of the immune system, but not cells of the neuroendocrine systems, can distinguish among closely related or truncated peptides (Blalock, 1989).

The interaction between the nervous and immune system is also evident from the cell types common to both systems: astrocytes-macrophages. Even if astrocytes are considered a type of macrophage, there are differences between these two cell groups. During inflammation, astrocytes are involved in interacting with invading T-cells. Activated T-cells are capable of passing the blood-brain barrier and thus infiltrate the brain parenchyma. In the CNS, T-cells can recognize peptides presented by astrocytes via MHC class II. However astrocytes are not as efficient as splenic antigen-presenting cells to activate Th1 and Th2 cells, and astrocytes are more effective in restimulating Th2 cells

than Th1 (Aloisi et al. 1998). Astrocytes, even if they belong to the macrophage cell group, play an important role in nerve regeneration and function. Astrocytes are the major source of nerve growth factor (NGF). NGF constitutes one of the members of the neurotrophin family, and it influences growth, differentiation, survival, and maintenance of peripheral and central neurons. NGF can also act as a protective agent in neuroinflammatory diseases (Oren et al. 2004). In addition to astrocytes, NGF can also be released by oligodendrocytes and microglia, and to a certain extent by Th1 cells (Santambrogio et al. 1994). Cells from both the nervous and immune system express the functional NGF receptor TrkA, suggesting that NGF participates in the interactions between these two systems. Astrocytes restimulate preferentially Th2 cells because these cells releasing IL-4 and IL-10 induce NGF secretion by astrocytes; while IL-2 and IFN-gamma released by Th2 do not stimulate NGF secretion (Oren et al. 2004).

The interaction between immune and neuroendocrine systems is more complex than expected because there may be a bidirectional relation among immune cells and behaviors. Mast cells can reside in the brain or they can migrate within the brain after inflammation. In mammals it has been demonstrated that the migration of mast cells in the brain can be triggered not only by inflammatory processes, but also by specific behaviors (Silver et al. 1996). For example in sexually active doves, after a brief period of courtship, there is a marked increase in mast cells in the medial habenula (brain) compared to control birds that have a relative absence of mast cells. During courtship there is an increase of gonadal steroids that could influence mast cell migration. In fact, exogenous administration or endogenous increase of gonadal steroids causes an increased number and activation level of mast cells in the brain. The number of mast cells remains

elevated also during the 4-5 week period when parents take care of the eggs and young. There is a reason why mast cells migrate specifically into the medial habenula: this structure in the brain is a major integrative link between septal nuclei and the midbrain.

Another example where mast cell migration and activity is influenced by behavior is seen in rats where stress was induced by social isolation (Silver et al. 1996). In stressed rats the mean number of mast cells in the thalamus was reduced by 90%. Sex hormones can directly and indirectly influence the synthesis and release of the granular content of mast cells. However, under these physiological conditions the secretion of granular content occurs without inflammation or tissue damage. Also, the interactions between mast cells and elements of the peripheral nervous system have been documented for peripheral ganglia, connective tissue, and gut mucosa. In these tissues and in the brain, mast cells could mediate alterations in blood flow, neurotransmission, and local immune responses such as switching of IgE in B-cells. Mast cells can produce up to 290 mediators, including histamine, interleukins 1-6, granulocyte-macrophage colony stimulating factor, a variety of proteoglycans, neuropeptides, nitric oxide, and chemoattractants for eosinophils and monocytes (Silver et al. 1996). A more clear effect of mast cells on behavioral changes is given in an experiment of induction of an anxiety status in rat (Ikarashi and Yuzurihara, 2002). In this experiment, several drugs that stimulate histamine release from mast cells and neurons, or that block histamine receptors with a histamine release inhibition effect, were used in combination and injected intracerebroventricularly. After 60 minutes from injection, the time spent in light areas and the number of times that rats crossed a dark-light boundary was recorded. It is known that anxious rats pass more time in the dark areas and will decrease the passages between

a dark-light boundary. The results of the experiment show that the release of histamine due to drug administration causes an increase in the anxious state of rats. The effect of the different drugs shows also that histamine can be released by mast cells or neurons.

Table 5-1. First appearance of immune organs of different fish species.

Species	Temp°C	Organ first appearance			Reference
		Thymus	Kidney	Spleen	
<i>Salmo gairdneri</i>	14	5 dprh			Grace and Manning, 1980; Tatner and Manning, 1983; Tatner, 1986
<i>Cyprinus carpio</i>	22	2 dph	2 dph	5 dph	Botham and Manning, 1981
<i>Danio rerio</i>	28	54 hpf	72 hpf		Trede et al. 2001; Willett et al. 1999
<i>Zoarcetes viviparus</i>		3 months	3 months	2 months	Bly, 1985

Abbreviations: dprh= days pre-hatch, dph= days post-hatch, hpf= hours post-fertilization.

Table 5-2. First appearance of lymphocytes in immune organs of different fish species.

Species	Temp°C	First appearance of lymphocytes				Reference
		Thymus	Kidney	Spleen	GALT	
<i>Salmo salar</i>	4-7	22 dprh	14 dprh	42 dph		Ellis, 1977
<i>Salmo gairdneri</i>	14	3 dph	5 dph	6 dph	13 dph	Grace and Manning, 1980; Tatner and Manning, 1983 Tatner, 1986
<i>Ictalurus punctatus</i>		5 dph	7 dph	14 dph		Petrie-Hanson and Ainsworth, 2001
<i>Cyprinus carpio</i>	22	3 dph	6 dph	8 dph		Botham and Manning, 1981
<i>Barbus conchonus</i>	23	4 dph	4 dph	7 dph		Grace, 1981
<i>Danio rerio</i>	28	65 hpf	3 weeks			Trede et al. 2001; Willett et al. 1999
<i>Zoarcetes viviparus</i>		2 mprep	1 mprep	After birth		Bly, 1985

Abbreviations: GALT= Gut Associated Lymphoid Tissue, dprh= days pre-hatch, dph= days post-hatch, hpf= hours post-fertilization, mprep= months pre-parturition.

Table 5-3. Time of maturation of the immune organs.

Development to mature lymphocytic organ					
Species	Temp°C	Thymus	Kidney	Spleen	Reference.
<i>Salmo gairdneri</i>	14	5 dph			Grace and Manning, 1980; Tatner and Manning, 1983; Tatner, 1986
<i>Ictalurus punctatus</i>		10 dph			Petrie-Hanson and Ainsworth, 2001
<i>Cyprinus carpio</i>	22	5 dph			Botham and Manning, 1981
<i>Danio rerio</i>	28	1 week			Trede et al. 2001; Willett et al. 1999

Abbreviations: dph= days post-hatch.

CHAPTER 6 FISH VACCINOLOGY

6.1. Fish Vaccine

The purpose of vaccination is to provide an individual with resistance to a particular pathogen without having to undergo a potentially risky infection. Vaccination confers a protection against disease, not necessarily infection. Thus, vaccines protect against outbreaks of disease, but not necessarily against persistent infection in an asymptomatic carrier state. Vaccination works by inducing a protective immune response that, through memory cells persists for a relatively long period of time, though the precise longevity varies. The term “immunity” refers to this memory state. This memory state varies among different pathogens, and also varies among fish species against the same pathogen. Natural exposure to infection acts as a booster to the immunity produced by vaccination, provided the memory cells are still present. In the absence of natural exposure, booster vaccination is necessary to maintain the level of immunity. Specificity and memory are two of the key elements exploited by vaccination since the adaptive immune response is stronger after the second encounter with antigen. The vaccine may stimulate non-specific defense mechanisms that provide a degree of protection over a short time, but these mechanisms lack a memory component.

Vaccines must be safe, have no side effects, and should induce a high level of protection. It is possible to achieve these characteristics using simple procedures such as heat or chemical inactivation of cultures of pathogenic micro-organisms; however, in

most cases, some degree of antigen purification or enrichment is necessary. In this last case it is important to identify the antigens that stimulate a protective immune response.

Vaccines can be classified as dead, live, and those that are genetically engineered. Dead vaccines are composed of inactivated pathogens or extracts. Live vaccines are composed of attenuated pathogens with low or no virulence. Live vaccines are developed by serial passage of a pathogen in culture, or by using naturally occurring mutants and cross-reacting strains, or using recombinant DNA technology deleting specific genes coding for virulence factors. Compared to dead vaccines, live vaccines produce a higher stimulation especially of the cellular branch of the immune system, and for this reason in general, they produce a greater immune response (Marseden et al. 1996). However, a problem associated with live vaccines is the possibility that the pathogen can revert to a virulent state or that it can still possess a residual virulence. In these cases, infection and mortality will be the result of fish exposed to the pathogen. Genetically-engineered vaccines are generally composed of a purified highly immunogenic antigen that has been expressed in a microorganism through insertion of DNA coding for that specific antigen. Generally, these vaccines provide superior protection because they are able to stimulate a more complete immune response resulting in a broader and more long-lasting immunity (Winton, 1998). Another significant advantage of genetically-engineered vaccines is the ability to construct multivalent preparations that can protect fish against several pathogens or different strains of the same pathogen.

All microorganisms and their products are composed of many different antigens, however, not all the antigens associated with virulence and pathogenicity are effective in stimulating an immune response. Poor immunogenicity of antigens in the host, especially

those associated with virulence, could be caused by an adaptation of the pathogen to avoid destruction by the host's immune response. Antigens may induce antibody production, but, unless these antibodies can neutralize the components of pathogenicity, they are valueless in giving protection. For this reason, assays of blood antibody levels following vaccination may give no indication of the degree of protection unless the antibodies are directed against pathogenic antigens. For example, when salmonids have been vaccinated with specific *Aeromonas salmonicida* bacterins, antibodies against the pathogen can be detected in the blood, but the fish are not protected (Ellis, 1985).

The form in which the antigen is administered may be very important. Generally, soluble antigens are administered in adjuvant to elicit antibody production. Oil-based vaccines have proven to give high levels of protection (Midtlyng et al. 1996a; Nordmo and Ramstad, 1997). In oil-based vaccines, it is important that the dispersed phase is homogenous to guarantee a reproducibility of injection. In the case of large oil droplets the dispersion tends to phase separate, with the possible sedimentation of droplets, and formation of a high-density lower phase or flotation of oil droplets. This will result in non-uniform dosing during the vaccination period. Phase separation can be avoided or minimized by producing vaccines with a small droplet size. Small droplet size is also preferable *in vivo* because fine dispersion will lead to an even distribution of the vaccine intracoelomically (Muller and Harnisch, 1998). There is also an optimum size for macrophage uptake and lymphatic absorption, the parameters determining vaccine efficacy and antigenic response. In *in vitro* studies, highest particle uptake from macrophages was observed with polystyrene model particles with a size of about 0.5-3 μm (Rudt and Muller, 1992). If the particle size is too large, the uptake is reduced

because the particles approach the size of the macrophage itself. Studies on lymphatic absorption of particulates demonstrate that optimum size is below 1 μm (Davis et al. 1993). Ideally, the vaccine dispersion should be stable after shaking the vaccine solution, at least for one day. In vaccine production, it is also important to reduce the viscosity of the formula. A low viscosity spreads more easily in the coelom and requires less injection force, thereby reducing the chances of possible lesions in the coelom. However, in some case, a lower viscosity may cause a too fast release of antigen, and a wider diffusion of the vaccine causing a too generalized inflammation.

The route of antigen administration is also important. Generally, intramuscular injection of antigen elicits higher antibody production than bath exposure (Fukuda and Kusuda, 1981; Midtlyng et al. 1996b). Another consideration is that the immunogenicity of antigens may vary among fish species.

In bacteria, serotype differences are frequently associated with variation in the structure of the lipopolysaccharides, LPS. If antigens which represent different pathogenic groups or strains are involved in eliciting a protective immune response, then the different serotypes must be incorporated into the vaccine to protect against the different serotypes of the pathogen. Such vaccines are called multivalent.

6.2. Adjuvants

Adjuvants are substances that enhance the immune response. They comprise a variety of compounds whose mechanisms of action are variable and in many cases not well understood. Administering antigen with adjuvant can considerably enhance the humoral immune response, especially to T-dependent antigens. Protection of fish against vibriosis and enteric red mouth disease can be effectively achieved without the use of

adjuvant, and it may be significant that in both cases the protective antigens are lipopolysaccharide, considered to be a T-independent antigen. Protection against other diseases has been achieved only when antigen was given in adjuvant (*Aeromonas* vaccine). In these cases the adjuvant may either improve the immunogenicity of the antigens or have a non-specific stimulatory effect on the non-specific defense mechanisms, or both (Ellis, 1988).

One of the problems in using adjuvants, is that they can cause side effects or severe local tissue responses (Lillehaug; Midtlyng, 1996; Midtlyng et al. 1996b).

The most effective adjuvant and one of the most widely used by experimental immunologists is Freund's Complete Adjuvant, FCA, which is a mixture of killed *Mycobacterium tuberculosis* in a mineral oil in which the antigen in saline solution is emulsified. The emulsion can only be administered by injection. FCA can cause undesirable side effects, such as local granuloma formation, autoimmune diseases, and tuberculin sensitization. By intramuscular injection, it induces sterile abscesses with extensive local muscle necrosis. Intracoelomic injection is less traumatic, but visceral adhesions and granuloma formation may result. Slight reduction in fish growth rate can also occur.

Freund's Incomplete Adjuvant, FIA, lacks the *Mycobacterium*. It is used in the same way as FCA and has only slightly less traumatic side effects, but it is not as effective an immunopotentiator as FCA.

Other injectable adjuvants are aluminum hydroxide gel mixed with saponin, certain oily emulsions, and potassium aluminum sulfate. Aluminum-based vaccines can be toxic to some species of fish or to small fish (Horne et al. 1984).

6.3. Injection Vaccination

The most effective way to immunize fish is by intracoelomic injection. This method permits the use of adjuvant that enhances the magnitude of the immune response. This method has some disadvantages, principally by requiring anesthetization and handling, both of which can be quite stressful for the fish. It is also very labor intensive and can be difficult to apply to small fish. Nevertheless, the overall smaller amount of vaccine required for this method, makes it more economical for fish larger than 40 g (Lillehaug, 1989).

Intramuscular injection on the dorsal surface of the fish is rarely used mainly because of the possibility of creating unsightly scarring, and because of potential leakage of the inoculum before absorption. However, salmon broodstock are usually injected intramuscularly in the “dorsal sinus”, a potential space between musculature in front of the dorsal fin (Mitchell, personal communication).

The swim bladder has also been used as a site for vaccine administration (Endo et al. 1995). In some species of fish, the swim bladder is surrounded by well-vascularized thin collagenous membranes that facilitate the absorption of the vaccine into the blood stream without causing as much damage to the body as intracoelomic or intramuscular injections. Moreover, the swim bladder has a large spaced lumen and the surrounding membrane can prevent the backflow and spread of the injected agents. Absorption of injected agents can take up to 7 days. This slow rate of absorption can be useful in some treatments against diseases, but it can also cause pathological damages to the organ wall. The swim bladder may be not recommended for physostomous fish due to possible vaccine leak through the connection to the esophagus.

6.4. Immersion Vaccination

The first methods developed for immersion vaccination required a hyperosmotic immersion step. The concept behind this technique was that a hyperosmotic media would have induced a greater absorption of antigen through the gills and skin of the fish. It was quickly realized that the hyperosmotic step was unnecessary and often very stressful. For this reason, direct immersion methods were developed. There are two main immersion methods, the dip and the bath. The substantial differences among the methods are the time of exposure to the antigen and the concentration of antigen in the water, the higher the concentration, the shorter the time. In dip vaccination fish are kept in the water with a vaccine dilution of 1:3 or 1:10 or 1:100 for 5-30 seconds. In the bath method fish are kept for an hour in the water with a vaccine dilution of 1:500-1:5000. A further variation of these methods is the spray method in which fish are sprayed with the vaccine after being removed from the water (Fukuda and Kusuda, 1981).

The uptake of some bacterial antigens, such as *Aeromonas* sp., by fish following immersion has been demonstrated to be quite effective. The mechanism of uptake is not precisely understood, but the gills appear to be the main route of entry, with the skin and lateral line system possibly playing a role. The failure to immunize fish against many diseases by the immersion method is probably not mainly associated with inadequate uptake of the antigens, but with the nature of the ensuing immune response. There is also the probability that mucosal immunity, and not systemic immunity, is produced. This hypothesis seems to be proven by the fact that while in injection vaccination there are high levels of circulating antibodies, in the immersion vaccination, very few antibodies are detected in the blood (Ellis, 1988; Lin et al. 2000). These results suggest that

immersion vaccinations stimulate integumentary immunity, which is quite sufficient to protect against natural exposure, but not to challenge by injection.

A disadvantage of vaccination by immersion compared to injection is that much more vaccine is required. In addition, immersion vaccinations done at high concentration can be toxic.

6.5. Oral Vaccination

Oral vaccination is a preferred method since it is suitable for mass administration to fish of all sizes, imposes no handling stress on the fish, and does not interfere with routine husbandry practices. Oral vaccination is the only method suitable for extensive pond rearing of fish where catching the fish prior to harvesting for injection or immersion vaccination is impractical. However, oral vaccination has some intrinsic limitations. In the case of killed vaccines large amounts are required, thus increasing the cost, and there is the uncertainty of individual dosage concentration since each fish consumes a different amount of food. Unfortunately, the most important limitation of oral vaccines is their poor potency, and many trials with many different vaccines have resulted in low or inconsistent levels of protection (Ellis, 1988). A major problem seems to be the destruction of antigens in the stomach and foregut before they reach the immune sensitive areas of the lower gut (Rombout et al. 1985). To overcome this problem, enteric-coated spheres can be used. Microparticles are used as carrier systems and they are capable of partially protecting and entrapping antigens against enzymatic degradation. Experiments with rats (O'Hagan et al. 1989) and with coho salmon (Piganelli et al. 1994) have shown that vaccination with microparticles confers a level of protection comparable to vaccination by intracoelomic injection.

Another reason for a low protection state following oral immunization is that oral vaccine may induce a mucosal, but not a systemic response. A semi-autonomous mucosal immune system exists in fish. Stimulation of any mucosa can lead to antibody production in all mucosal compartments (gills, skin, guts), without necessarily stimulating a systemic response and inducing a memory state. Induction of mucosal immune responses via the oral route often requires multiple oral administrations (Joosten et al. 1997; Lin et al. 2000; O'Hagan, 1992).

6.6. Biomolecular Vaccines

The application of biomolecular techniques to vaccine design can help produce vaccines that confer higher protection and are safer than vaccines produced by conventional techniques. In fish, research into recombinant DNA technology has focused mainly on viral vaccines. Currently, there are only a few vaccines available, including one vaccine against Infectious Pancreatic Necrosis Virus (IPNV) (Leong, 1993; Leong and Fryer, 1993; Leong et al. 1997; Lorenzen, 1999; Midtlyng, 1997). Expression-based vaccines can be divided into several different groups: 1) those agents that replicate within the host, such as attenuated strains; 2) recombinant viral and bacterial vectors; and 3) nonreplicating agents that serve only as vehicles to deliver nucleic acid sequences, such as retroviral sequences and genetic immunization.

The first step in the production of a recombinant DNA vaccine is to identify the proteins/antigens of the pathogen that stimulate a protective immune response in the host. More than one antigen may be protective, and some antigens may stimulate some parts of the immune system more than others. Then, the DNA coding for the protective antigen is inserted into a suitable microorganism (virus, yeast, or *Escherichia coli*) that is easily

cultured so the protective antigens can be produced on a large scale. Because no infectious agent is present, these types of vaccines have a high level of safety, and low production costs after initial research and development (Winton, 1998).

Bacterial expression systems based upon *E. coli* may lack the ability to glycosylate and correctly fold some types of proteins, resulting in less effective protection. In these cases another expression system that is able to produce a protein in its correct native conformation, such as baculovirus vector growth in insect cells, must be chosen. However, these vectors can make the vaccines too expensive. These alternative expression systems were used to produce a vaccine for rainbow trout against the viral haemorrhagic septicemia virus (VHSV) (Lecocq-Xhonneux et al. 1994). The cloned gene for one virus glycoprotein was inserted into a baculovirus expression system and the recombinant protein was produced in insect cells. The recombinant protein had a slighter lower molecular weight than the native viral protein and displayed a different pattern of glycosylation. The recombinant protein was transported to the plasma membrane and induced cell fusion in a manner similar to the authentic protein. The vaccine based on this recombinant protein conferred a protection to rainbow trout similar to that conferred by killed and attenuated vaccines, and neutralizing antibodies recognized it.

With recombinant DNA technology, besides inserting sections of DNA into the genome of a cell, it is also possible to delete sections. Specific deletion of a gene that codes for a virulence factor in a pathogen, while preserving other protective antigens, allows the production of attenuated forms where reversion to virulent wild type is impossible. This technique is valuable in production of live vaccines.

In addition to traditional genetic immunization and recombinant DNA technology, there are several other biomolecular techniques that have been applied to vaccine production.

Genetic immunization using naked DNA is the most recent approach in vaccine design. This technology is based on the observation that skeletal muscle cells injected with purified plasmid DNA express plasmid-encoded proteins. In mammals the specific immune response after DNA vaccination is caused by antibodies, T-helper cells and cytotoxic cells.

Protein engineering. The aim of this technique is to improve immunogenicity of an antigen or to mass produce it cheaply. Protective antigens may be made more immunogenic by cleavage of epitopes that stimulate the T-cell suppressor. The protective epitopes of an antigen may be composed of a short sequence of amino acids that can be artificially synthesized quite cheaply for use as vaccines in combination with appropriate carrier antigens.

Anti-idiotypic vaccines. These are antibodies, the antigen-binding site of which mimics the structure of a protective antigen. They are produced by first raising antibodies to the appropriate antigen in a suitable animal. The specific antibodies produced (idiotypes) are then used to raise further antibodies that react with the antigen-binding site of the first antibody (anti-idiotypic antibodies). The antigen-binding site of the anti-idiotypic can be used as a vaccine since it resembles stoichiometrically the original antigen and will induce formation of antibodies which cross reacts with it. Such vaccines have advantages when protective antigen is difficult to obtain in large amounts or produces undesirable side effects.

6.7. Genomics and Proteomics Applied to Vaccine Design

New approaches to vaccine development are important because there are several infectious diseases for humans and animals for which the traditional approaches have failed to produce effective vaccines. Moreover, especially for humans, regulatory authorities often require that vaccines meet high standards of safety. For these reasons, there is increased interest in the application of the new technologies known as genomics, proteomics, and transcriptomics to human and animal vaccine design.

Genomics, proteomics and transcriptomics have become important tools for increased understanding of the biology of pathogens (viral, bacterial and parasitic) on a large scale, giving information on which genes and proteins are expressed and under which conditions. This information can help assign functions to unknown genes and proteins, decipher the mechanism of pathogenicity of organisms, reveal new possible targets for drugs, and assist in the design of new vaccine candidates for humans and animals (Barret et al. 2000; Betts, 2002; Cordwell et al. 2001; Grandi, 2001; Klade, 2002; Moxon and Rappuoli, 2002; Nilsson, 2002; Potter and Babiuk, 2001; Tarleton and Kissinger, 2001; Teixeira et al. 2002; Wallace and Wallace, 2002). For example, antigens present only in virulent bacteria strains may make good vaccine candidates.

DNA microarrays are one of the most useful tools for the study of the transcriptome, the complete set of transcripts produced by an organism. In this technique DNA elements are arranged as spots in a grid pattern on a solid support. These arrayed targets are hybridized with complex probes prepared from an RNA population for a particular cell type or tissue. The most common format involves PCR products specific for each open reading frame (ORF) in the genome attached to a glass microscope slide.

Gene expression profiling is performed by two-color hybridization. DNA labeled with different fluorochromes is produced from RNA isolated from two experimental conditions. The two populations are combined in equal quantities and hybridized to the array. Scanning the array gives the quantitative fluorescence intensities for each fluor in each spot, from which ratios are calculated to give relative expression levels for each gene. This analysis can reveal even shifts of hundreds of genes expressed. Such techniques have been used to study the whole genome of *Streptococcus pneumoniae* to identify suitable antigens for vaccine production (Wizemann et al. 2001).

Through exploring the effects of drug action using DNA microarrays, it is possible to identify genes within pathways that may have not been previously realized, and may provide alternative drug targets. Moreover, the generation of signature profiles of drug action may allow prediction of the mode of action of novel compounds. Improvements in the sensitivity of this technique may be achieved through amplifying mRNA, thus facilitating analysis of small amounts of RNA recovered from tissues or cells.

Another technique for studying genome expression is In Vivo Expression Technology (IVET). Plasmid libraries are created with random fragments of bacterial chromosomes fused to promoterless genes that encode an antibiotic resistance gene. A single crossover between the plasmid and the corresponding homologous region on the chromosome produces mutant bacteria. The bacteria are then injected in to antibiotic treated animals. At this point only the mutant bacteria that have the antibiotic resistance gene inserted in to their chromosome survive. Virulence genes are identified analyzing the surviving bacteria clones. This technique has been used to identify virulence genes in

Salmonella typhimurium, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Vibrio cholerae* and *Staphylococcus aureus* (Grandi, 2001).

In Signature Tagged Mutagenesis (STM), bacterial mutants are created by random insertion of a set of transposomes differing by a short synthetic nucleotide sequence (TAG), so that each mutant can then be recognized by its TAG. The bacteria mutants are then injected in to animals and the bacterial clones with a mutation in a gene needed for pathogenesis will not survive. The missing mutant clones can be detected by recovering bacteria and doing a TAG-specific DNA hybridization, in this way all the genes required for infection can be identified. For example, in a study on Group B *Streptococcus*, 120 out of 1600 transposome mutants were defective for survival in the animal host, and only 50% of these mutants have insertions in known genes (Jones 2000).

Proteomics, in addition to describing the protein complement to the genome, can provide important information on proteins expressed at defined points in time or under defined conditions, their location within the cell, and the existence of post-translational modifications. Protein analysis can be more useful than mRNA analysis for several reasons. The final products of genes are proteins; mRNAs can be present in a cell but not translated; and from a single mRNA molecule, several proteins can be produced, as in the case of some hormones such as calcitonin and calcitonin-related gene. Both hormones are translated by the same mRNA, but by different splicing occurring in the brain or in the thyroid. The simple analysis of mRNA would not detect all these different proteins produced by the same mRNA. Stability, half-life, post-transcriptional, co-translation and degradative modification of proteins suggest that mRNA abundance and response to

environmental stimuli may not completely represent the expression of a corresponding protein *in vivo*.

Protein analysis, however, can present some difficulties that are not encountered in genome analysis, such as target abundance and molecular recognition. Another difference is that target DNA or RNA can be amplified by PCR. There are no similar techniques that easily allow amplification of target proteins. In genome studies, sequences can be recognized by oligonucleotide probes, but for protein analysis, recognition is mediated by antibodies, other proteins or ligands, and each of them is very specific for only one or a few proteins.

In proteomic analysis, several techniques are utilized, with the most common being two-dimensional gel electrophoresis (2DE). Two-dimensional gel electrophoresis is a technique that can be applied to a complex protein mixture, such as that extracted from whole cells and tissues. The proteins migrating in a pH gradient gel are first separated based on their charge (isoelectric point). Then, the proteins migrate according to their molecular mass, in a direction perpendicular to the first migration axis. Size fractionation is achieved by first equilibrating the isoelectric focusing gel in a 2% solution of the detergent sodium dodecylsulphate (SDS) which binds non-specifically to all proteins and confers a uniform negative charge. After fractionation, the protein gel is stained using, for example, silver staining or Coomassie brilliant blue or non-covalent fluorescent dyes. The outcome is a unique pattern of dots, each dot representing a protein. This pattern can be used as a protein fingerprint of the cell. Typically more than 1,000 distinct proteins can be separated in a standard gel, although the most sophisticated systems allow for the

separation of up to 10,000 protein spots. Western blotting of the gel and detection of antigens with patient sera can help distinguish immunorelevant proteins.

This technique can be utilized to easily compare protein expression of a particular cell in different situations by just comparing the 2DE gels and looking for a spot absent or present in one of the gels. However, a problem for this type of analysis is that the conditions in which the gels are prepared and run should be the same in each experiment, and this can be difficult to accomplish.

To enhance separation of proteins, instead of 2DE, reverse phase microcapillary liquid chromatography can be utilized. Multidimensional chromatography can also be applied. This technique consists of serial chromatographic separation according to different properties.

Identification of proteins on the 2DE gel can be achieved. First, proteins inside the gel are digested with trypsin. The resultant peptide mixtures can be analyzed with mass spectrometry, measuring the mass of each peptide obtained from the digestion of the same protein. Each protein produces a characteristic peptide mixture that can be used as a fingerprint. The measure of the mass of the peptide can be correlated to the amino acid composition of each peptide, and then the amino acid sequence of the protein can be determined. This fingerprint can be compared with theoretical digests of proteins in databases through a bioinformatics tool in order to identify the protein. Otherwise, peptides can be separated by size-exclusion chromatography (SEC) and reversed-phase (RP) chromatography, then directly analyzed by mass spectrometry (MS).

One problem of this technique is sensitivity. Lack of recovery of a given protein in 2DE gel does not necessarily mean that the protein is not expressed. In fact, the

resolving power of 2DE in conjunction with narrow-range immobilized pH gradients and subcellular prefractionation has been estimated to reveal up to 75% of protein genome, while the remaining 20-30% cannot be resolved due to technical limitations associated with this technology. Moderate or low abundant proteins could not be observed in the 2DE, especially if a nearby spot representing a high abundant protein obscures their signal. Scarce proteins may be completely undetected. Fractionating the total protein sample and running a gel specific for particular types of proteins, such as membrane proteins or proteins with a particular isoelectric pH, can overcome this problem.

Prefractionating complex mixtures into specific cellular compartments, such as membrane or extracellular or intracellular proteins, is important because for pathogenic organisms, proteins of interest are most likely confined to the membrane or extracellular fractions. However, due to their intrinsically hydrophobic nature, membrane proteins can be difficult to solubilise in gel. Recently, several new solubilising agents have been developed and have allowed the visualization of significantly more hydrophobic membrane-associated proteins (Cordwell et al. 2001). This type of analysis was performed on *Streptococcus agalactiae* to identify the major outer surface proteins that can be used for vaccine production (Hughes et al. 2002).

Bacterial proteins secreted during growth may be important for several pathogenic processes, including toxicity or cell-signaling, but the proteomic analysis of such proteins can be difficult due to the presence of contaminating proteins from undefined sources or from supplement animal sera. In some bacteria, the analysis of such proteins could be very important for vaccine production. For example, relevant immunogenic antigens

important for vaccine production were identified analyzing the secreted proteins of group A *Streptococcus* (Lei et al. 2000).

Another technology used to study the proteome of a target cell or tissue is protein arrays. Protein arrays are in theory very similar to DNA arrays being miniature devices that have proteins or molecules that recognize other proteins. However, protein microarrays have more limitations than DNA microarrays. First there is no procedure that amplifies the sample proteins as there is for DNA. Second, while all DNA sequences are constituted by the same four nucleotides that are similar in their chemical properties, proteins are comprised of 22 amino acids with different chemical properties. The principles of molecular recognition are very different among amino acids and also vary among different proteins. For this reason the same reaction conditions can never be applied for all proteins. Third, in a DNA array, labels are incorporated evenly and do not interfere with hybridization. Fourth, solid substrate, such as nylon or glass, does not interfere with hybridization. In contrast, for proteins, labeling is much more variable and both labeling and attachment to a substrate may interfere with protein binding by affecting protein folding or by blocking the binding site. In these arrays, antibodies are attached to the array or chip surface.

Antibody arrays can detect specific proteins. Because antibodies are highly discriminatory, they are suitable for detailed analysis of protein profiles and expression levels. There are three different types of antibody arrays. The first is a standard immunoassay in which the antibodies are immobilized and are used to capture labeled proteins from the solution. Protein expression levels are quantified by measuring the signal, typically fluorescent, that has been incorporated into the proteins. This technique

allows exposure of two different protein samples at the same time, each labeled with a different fluorophore. The second technique consists of a miniature sandwich assay in which unlabelled proteins are captured from the solution by antibodies on the array after which the proteins are detected by a secondary antibody. This technique requires that the two antibodies recognize different parts of the proteins. The advantage of this technique is that it is not necessary to label the proteins, a process that is variable and sometimes inefficient. The third technique involves a tertiary detection system, and therefore it is more sensitive. The principle of this technique is that the immobilized antibody captures a protein. After, a secondary antibody linked to a circular oligonucleotide will recognize the protein. In the presence of a circular DNA template and dNTPs, DNA polymerase will amplify the circular DNA template producing a long DNA concatamer containing hundreds of copies of the template. Then a fluorescent-labeled oligonucleotide probe can be added to detect the DNA template.

More recent techniques in proteomics use a microfluid device instead of a gel. These devices should have a higher sensitivity because of the faster kinetics of binding in solution than in a solid phase, and they should have a higher throughput because the arrays are constructed in three dimensions.

Another technique to identify a new possible targets for vaccine production is in silico analysis. This technique employs algorithms involving known gene sequences to predict genes encoding secreted, surface-associated antigens and virulence factors. This technique analyzes the genomic sequence of pathogens to detect proteins with transmembrane regions, a secretory leader sequence, or tandem repeats at 5' ends of genes. Tandem repeats at the end of genes are often found in virulence genes. New

virulence factors can be detected by searching for unknown genes that are co-regulated with known virulence genes. The selected genes are expressed in *E. coli* and the expressed proteins are purified and tested for their effect on the immune system. This technique is very fast because it uses robotic stations. Five scientists can produce and purify 100 proteins each month. However, a limitation of this technique is that it can not be used to detect new families of virulence factors. In a study on *Chlamydia pneumoniae*, 147 membrane-associated proteins were identified, 91% of them were successfully expressed in *E. coli*, and 58 proteins induced Igs that bind *C. pneumoniae* (Grandi, 2001).

Collectively, these technologies will allow the identification of most of the antigens targeted by the immune system under certain pathological situations. Once the target antigens are identified, their genes must be cloned and expressed, then the synthesized proteins must be purified and tested *in vitro* and *in vivo* for identification of candidates for vaccine production. Moreover, the comparison of multiple immunomes may allow the discovery of immunogenic structural features shared and conserved among different pathogens, which could form the basis of broadly protective vaccines.

6.8. Herd Immunity

Concomitant with research on vaccine development, there is a need for an improved understanding of how best to use vaccines to protect the community as well as the individual. Epidemiological and economic issues are at least as important as technological ones (Anderson and May, 1985; Waltner-Toews, 1989).

Herd immunity is the ability of groups of animals to resist becoming infected or to minimize the extent of infection, which is related to a decrease in the probability of adequate contact between infected and non-infected individuals.

The persistence of an infectious disease within a population requires that the density of susceptible individuals exceed a critical value such that, on average, each primary case of infection generates at least one secondary case. It is therefore not necessary to vaccinate everyone within a community to eliminate infection, but it is sufficient to vaccinate a percentage of the population so as to reduce the susceptible fraction below the critical point. The central problem for the epidemiologist then is to calculate the proportion of the population that should be vaccinated. This calculation is not easy because many factors must be considered, such as desired level of vaccine protection, primary infection rate, transmission rate, incubation time, duration of acquired immunity, age-related changes in infection susceptibility, best age for immunization, genetic factors, population density, and behavioral factors. For example a more dense population requires higher rates of vaccination (Anderson and May, 1985; Waltner-Toews, 1989).

When the concept of herd immunity is kept in mind, it becomes clear why the use of vaccines that confer low or moderate protection is still beneficial. In fact, these vaccines may slow down or stop the rate of disease transmission. A reduction in the rate of transmission may be helpful especially when the production periods are not too long, as in aquaculture where the production period is generally of one or two years.

CHAPTER 7 IMMUNOSTIMULANTS

7.1. Immunostimulants and Experiment Design

The term immunostimulants is applied to several compounds of diverse chemical structure that have been shown to stimulate the immune system (Anderson, 1992; Raa, 1996; Sakai, 1999). The exact mechanisms of the immune system stimulation are still not well understood, and there are still doubts on the real efficacy of some of these compounds. For many immunostimulants, such as vitamin C, different investigations have shown diverse and even conflicting efficacy results. However, comparing the results of different studies is not an easy task due to differences in experimental protocols including variations in doses, methods, and length of administration.

During experimental analyses, the body's response to immunostimulant administration over time is often not considered. This class of compounds can stimulate the immune system, but prolonged dosing will produce a homeostatic response with consequent decreases in stimulation. In addition, different immune parameters can be stimulated at different doses and times. One example of these issues is evident in research examining vitamin C administered to gilthead seabream (*Spaurus aurata*) (Ortuno et al. 1999). In this study, fish were fed diets containing 0.5 and 3 g/kg ascorbic acid for 2, 4, 6, 8 and 10 weeks. Some of the non-specific immune parameters were enhanced by dietary intake of vitamin C, but they peaked at different times. Leukocyte phagocytic ability and capacity in head kidney increased after 2 weeks, natural hemolytic complement activity after 6 weeks, and leukocyte respiratory burst after 8 weeks, but leukocyte migration was

not affected. After 8-10 weeks, the serum complementary activity was almost the same in all the trials. Even when high levels of ascorbic acid were maintained in the serum, the immune responses did not improve, but returned to their normal levels. These data suggests that after a transient enhancement of the non-specific immune response as a result of vitamin C supplementation, an adjustment of the immune response to the new conditions of higher ascorbic acid availability occur. If this hypothesis is correct, it might explain the absence of effect described in some previous studies with this vitamin when long administration periods were tested.

Body storage capability for different immunostimulants is another consideration when experimental results are analyzed. In studies where different doses of an immunostimulant that can be stored in the body, are tested, lack or a non-significant difference in results among diets may be due primarily to body storage of the compound. For this reason before the experiment it is important to feed diets without immunostimulant.

Additional factors include storage capabilities and retention period. These may differ, for a particular compound, among organs. For example, in rainbow trout after the fish were switched for two weeks to the control diets, plasma and liver ascorbic acid levels were strongly reduced meanwhile the level was still high in the head kidney. The longer retention of vitamin C in the head kidney is related to its lymphoid function: leukocytes are able to store elevated amounts of ascorbic acid in their cytosol and, therefore, require a longer period of time to become depleted (Verlhac et al. 1996).

7.2. Vitamin C

Ascorbic acid is involved in many physiological functions including growth,

development, reproduction, wound healing, metal ion metabolism, response to stressors, immune response, and perhaps lipid metabolism through the action of carnitine synthesis.

Most teleost fish do not synthesize vitamin C because they lack the enzyme L-gulonolactone oxidase. This vitamin is synthesized in other phylogenetic groups of fish, such as sea lamprey, *Petromyzon marinus*, Atlantic dogfish, *Squalus acanthias*, sterlet sturgeon, *Acipenser ruthenus*, white sturgeon, *Acipenser transmontanus*, paddlefish, *Polyodon spathula*, and in some teleost such as Atlantic halibut, *Hippoglossus hippoglossus*, and Atlantic salmon, *Salmo salar*, (Dabrowski, 1994; Dabrowski and Clereszko 2001; Maeland et al. 1999; Maeland and Waagboe, 2000; Moreau, 1999).

Signs of vitamin C deficiency in fish include decreased growth, scoliosis, lordosis, internal and fin hemorrhages, distorted gill filaments, fin erosions, anorexia, and increased mortality (Halver et al. 1969; MacConnell, and Barrows, 1993).

Organ and tissue concentrations of ascorbic acid are correlated to dietary intake levels (Hardie et al. 1991; Verlhac et al. 1996). Brain, thymus, head kidney, and leukocytes concentrate more vitamin C and maintain its level for longer periods than other organs, such as liver, in cases of dietary deficiencies. Liver and head kidney are the primary storage organs for vitamin C in fish. The high level of vitamin C found in thymus, brain, head kidney, and leukocytes may be related to the requirement for antioxidant substances to maintain cellular membrane integrity to preserve vital organs from oxidative processes, and to allow the optimal functioning of immune cells. The high level of this compound in the head kidney could also be related to the lymphopoietic function. Chromaffin cells, responsible for catecholamine biosynthesis, are present in trunk kidney. That could explain the high level of ascorbic acid present in this organ.

Trunk kidney and spleen are also able to store large amounts of vitamin C.

Vitamin C is involved in the synthesis of cortisol and catecholamine hormones involved in the stress response. In a stressed state, plasma cortisol levels increase and the immune system efficiency decreases. Cortisol causes changes in numbers and proportions of circulating leukocytes, generally resulting in lymphocytopenia and neutrophilia. Cortisol also has an effect on the non-specific immune system causing inhibition of respiratory burst activity, phagocytic activity, and leukocyte migration. Cortisol also decreases the mitogen response and antibody production (Barton and Iwama 1991). Vitamin C can improve the immune system by acting as a brake on steroidogenesis through peroxidation of unsaturated fatty lipids and so preventing their conversion into cholesters that are important components of the cortisol. For this reason, during stressful situations, the requirement of vitamin C increases.

Vitamin C plays a significant role in the immune response and resistance to infectious diseases primarily by stimulating components of the non-specific immune system, including the complement pathway, serum lysozyme, macrophages, and neutrophils phagocytic activity (Hardie et al. 1991; Li and Lovell, 1985; Ortuno et al. 1999; Roberts et al. 1995; Verlhac et al. 1996). During phagocytosis, immune system cells produce reactive oxygen radicals. These compounds have potent microbicidal activity, but they are also auto toxic to fish macrophages (Secombes et al. 1988). Administration of ascorbic acid has been shown to increase survival of fish challenged with pathogens. Increased survival was observed in salmon challenged with *Aeromonas salmonicida* (Hardie et al. 1991); in channel catfish challenged with *Edwardsiella tarda* (Durve and Lovell, 1985) and *E. ictaluri* (Li and Lovell, 1985); in Indian major carp,

Labeo rohita, challenged with *E. tarda* (Sahoo and Mukherjee, 2002); in rainbow trout challenged with the ciliate parasite *Ichthyophthirius multifiliis* (Wahli et al. 1986).

However, in several other studies, supplementation of vitamin C did not improve the immune system or the resistance of fish to bacteria or parasites (Bell et al. 1984; Lim et al. 2000; Lygren et al. 1999; Sealey and Gatlin, 2002). In channel catfish, administration of vitamin C increased macrophage migration, but did not increase survival against *E. ictaluri* (Lim et al. 2000).

Ascorbic acid is also involved in mineral metabolism, influencing absorption and decreasing metal toxicity. Vitamin C transforms metals, such as cadmium, nickel, lead, into their reduced forms, which are less efficiently absorbed and more rapidly excreted.

7.3. Vitamin E

Vitamin E, acting as an antioxidant, protects biological membranes and assists in optimal functioning of the immune system. Phospholipids of mitochondria, endoplasmic reticulum, and plasma membranes possess special affinities for alpha-tocopherol. Being a lipid soluble antioxidant, vitamin E protects the highly unsaturated fatty acids in cells against oxidative degeneration by terminating free-radical-initiated chain reactions and decreasing the production of lipid peroxides and reactive oxygen species. Because these chemicals are autotoxic and can destroy neutrophils and macrophages, they can adversely affect the immune system (Lall and Olivier, 1991). Alpha-tocopherol also stimulates the phagocytic response, and total superoxide dismutase activity in liver and muscle (Lygren et al. 1999). Vitamin E significantly increases immunity and decreases mortality in Indian major carp challenged with *E. tarda* (Sahoo and Mukherjee, 2002).

Vitamin E deficiency results in decreased T and B lymphocyte function and phagocytic index in rainbow trout challenged with *Yersinia ruckeri* (Blazer et al. 1984), coelomic macrophage function in rainbow trout (Blazer, 1982), as well as serum complement function and serum capability to opsonize bacteria in Atlantic salmon (Hardie et al. 1990; Montero et al. 1999).

In some studies no positive response on the immune system was observed with administration of vitamin E (Li et al. 1993; 1998) nor was there resistance to bacterial challenge (Lall et al. 1988; Sealey and Gatlin, 2002).

7.4. Vitamin A

Vitamin A and other carotenoids can influence the innate and specific immune responses. These compounds have high affinity for toxic oxygen radicals, which result mainly from lipid peroxidation, but which are also generated during the respiratory burst of white blood cells in the initial response to an infection (Benedich, 1989). For this reason the carotenoids can enhance the ability of macrophages, killer T-cells and cytotoxic T-cells to kill tumor cells and bacteria. This capability is demonstrated in homeothermic animals where β -carotene and canthaxanthin can enhance B and T-cell proliferation *in vivo* (Benedich and Shapiro, 1986). However the effect of these compounds on the immune system of fish has not been well investigated.

In a study on rainbow trout, vitamin A and astaxanthin were shown to have only limited potential as immunomodulatory agents (Thompson et al. 2001). Fish fed diets deficient in vitamin A and astaxanthin presented lower serum antiprotease and classical serum complement activity, and in a diet deficient in vitamin A, leukocyte migration was decreased. The total serum immunoglobulin level, serum lysozyme activity, and

phagocyte respiratory burst activity were unaffected by astaxanthin or vitamin A deficient diets. The results of this research showed that deficiencies of vitamin A and astaxanthin can cause a decrease of serum antiprotease activity and this could affect the resistance of fish to external pathogens. In fish, the antiproteases play an important role in neutralizing the extracellular proteases of bacterial pathogens, and α 2-macroglobulin level in particular has been reported to be an important correlate of protection against infection by *Aeromonas salmonicida* (Ellis, 1985).

7.5. Folic Acid

The effect of folic acid on the immune system was investigated in channel catfish (Duncan and Lovell, 1994). Deficiency of folic acid caused binucleated erythrocytes, spectacle cells (abnormally dividing erythrocytes with two nuclei joined by a strand of nuclear material), hemocytoblasts (immature blood cells), cytoplasmic clearing, poikilocytosis (variation in shape of erythrocytes), pyknosis (condensation of the nucleus of erythrocytes), smudge cells (disintegrating blood cells), macrocytes, erythrocytes with irregular nuclear borders, and segmented or spreading neutrophils. In the same research it was observed that fish challenged with *E. ictaluri* showed maximum survival and antibody production when folic acid was fed with a high level of vitamin C, but only high supplementation of folic acid improved survival when the diet contained a marginal level of vitamin C. In contrast, in Nile tilapia, *Tilapia nilotica*, administration of folic acid did not increase antibody levels nor did it improve survival of fish challenged with *Streptococcus iniae* (Lim and Klesius 2001).

7.6. Iron

Iron is an essential element for the functioning of organs and tissues of higher animals, including fish, because of its important role in oxygen transport and cellular respiration. Iron is also one of the most important micronutrients for its effect on immune system functions and host defense against infection. Chemotactic response of coelomic macrophages to *Edwardsiella ictaluri* exoantigen was depressed in iron-deficient channel catfish (Barros et al. 2002; Lim and Klesius, 2001; Sealey et al. 1997), but this abnormality was remedied by feeding an iron repleted diet for 4 weeks (Lim and Klesius, 2001). However, a high level of supplemental iron may be harmful to fish due to the delicate balance between the requirement of iron for host defense mechanisms and the requirement of iron to sustain microbial growth (Sealey et al. 1997).

Fish can absorb soluble iron from the water across the gill membrane and intestinal mucosa, but feed is considered the major source of iron for fish due to low concentrations of soluble iron in natural waters (Lim et al. 2000). The total dietary iron requirement for optimum growth, feed efficiency, hematological values, and immune response of juvenile channel catfish has been determined to be about 30 mg/kg diet (Sealey et al. 1997).

Several studies have examined the combined effects of vitamin C and iron supplementation on fish immune system function because ascorbic acid is known to be involved in the metabolism of iron in animals. Vitamin C enhances the absorption of iron from the intestine by reducing ferric iron to ferrous state, a more soluble form that is readily absorbed. Ascorbic acid is also involved with adenosine triphosphate (ATP) in the release and reduction of the ferric iron from ferritin, and its subsequent incorporation

with iron-binding proteins, apoferritin and transferritin, into tissue ferritin. High levels of dietary ascorbic acid in the absence of dietary iron may accelerate iron deficiency and increase the severity of microcytic anemia in channel catfish (Lim et al. 2000).

Significant interactions between dietary levels of ascorbic acid and iron were observed in channel catfish with regard to hematological values and survival of fish challenged with *E. ictaluri* (Lim et al. 2000). Mean macrophage migration in the absence or presence of *E. ictaluri* exoantigen was significantly higher for fish fed the iron supplemented diets, but neither dietary level of iron nor ascorbic acid nor their interaction influenced the survival level of juvenile channel catfish against *E. ictaluri* challenge. However, the onset of mortality was earlier for fish fed the iron-deficient diet.

7.7. Selenium

Selenium is an essential trace element for all animals. It is a component of enzymes such as glutathione peroxidase (GSH-Px). This enzyme catalyzes reactions necessary for the conversion of hydrogen peroxide and fatty acid hydroperoxides into water and fatty acid alcohols by using reduced glutathione, thereby protecting cell membranes against oxidative damage. GSH-Px acts along with vitamin E as a biological antioxidant to protect polyunsaturated phospholipids in cellular and subcellular membranes from peroxidative damage. Tissues or cellular components that are inherently low in glutathione peroxidase will not be affected by selenium, but will be still protected by vitamin E, which acts as an antioxidant by a mechanism not involving GSH-Px. Wang (1996) found that selenium deficiency in the presence of dietary vitamin E seriously reduced GSH-Px activity in channel catfish and that Se-Methionine was a more potent source of selenium for GSH-Px than sodium selenite. A possible role of GSH-Px in the

immune response is protecting cell membranes against peroxide damage during phagocytosis. Production of hydrogen peroxide in phagocytes is necessary in the process of killing microbes. However, the overproduction or leakage of peroxides into cytoplasm can damage the cell membranes of macrophages when GSH-Px activity is low.

Selenium also has a protective effect against the toxicity of heavy metals such as cadmium and mercury. In addition, selenium is important for normal humoral and cellular immune response. Selenium plays a role in antibody production through the proliferation and protection effects of GSH-Px on B-lymphocytes. Suppressed antibody production appears to be a common result of selenium deficiency among animals. Chinook salmon fed a selenium deficient diet showed greater mortality from *Renibacterium salmonarium* infection (Thorarinsson et al. 1994).

The concentration and source of selenium can influence the immune response to bacterial challenge (Wang, 1996; Wang et al. 1997). In channel catfish dietary selenium concentrations for maximum survival from *E. ictaluri* challenge was 0.20 mg/kg for fish fed Se-Methionine, and 0.40 mg/kg for fish fed Se-Yeast (Se-Y) or Na₂SeO₃. Antibody production generally increased as dietary concentration of selenium increased. Antibody titer was highest for fish fed Se-Y, intermediate for fish fed Se-M and lowest for fish fed Na₂SeO₃. Macrophage chemotactic response was higher for fish fed Se-M and Se-Y than for the control and Na₂SeO₃. A possible explanation for these results is that amino acid bound trace minerals have higher biological values than inorganic forms because the elements remain chelated after intestinal absorption, are transported intact to target tissues, and consequently are more available for metabolic processes than inorganic forms. However, this possible explanation remains to be proven.

Supplementation of vitamin E or selenium to channel catfish at higher doses than the recommended levels of one or both nutrients may enhance macrophage functions (Wise et al. 1993). However, selenium and vitamin E do not complement each other, and neither compensates for a deficiency of the other. Megadoses of selenium used for disease therapy may involve risks because of the high toxicity of this mineral to animals.

Fish can absorb selenium present in the water across the gills and digestive tract (Lim et al. 2001). The uptake of selenium as selenite across the gills is very efficient even at low waterborne concentrations. At least in rainbow trout, the level of selenium in the water can affect the dietary selenium requirement (Hodson and Hilton, 1983).

Selenium has been found to be required in the diet of Atlantic salmon, rainbow trout and channel catfish (Lim et al. 2001), with the actual requirement dependent upon the source ingested, polyunsaturated fatty acids, vitamin E content in the diet, and the concentration of selenium in the water.

7.8. Zinc

Zinc is required for normal growth, development and function of all animal species. The primary functions of zinc are based on its role as a cofactor in several enzyme systems and as a component of a large number of metalloenzymes. Zinc plays an important role in immune defense mechanisms. Zinc is fundamental to T-cell function and a deficiency leads to thymus atrophy and decrease thymic hormone and lymphokine production in mice (Fraker et al. 1977). Zinc may also affect resistance to parasites and bacteria. For example, mice fed a zinc deficient diet for 8 days were significantly more susceptible to the parasite *Trypanosoma cruzi* than mice fed adequate zinc (Fraker et al. 1982). Channel catfish fed a diet deficient in zinc had significantly higher mortality from

Aeromonas hydrophila or *E. ictaluri* infections than fish fed diets replete with zinc (Paripatananont and Lovell 1995; Scarpa et al. 1992). Similar results were observed with zebrafish, *Danio rerio*, exposed to *Listeria monocytogenes* (Rougier et al. 1992). However, in the research of Lim et al. (1996) dietary zinc did not decrease mortality in channel catfish infected with *E. ictaluri*, even though the relative percentage of neutrophil and macrophage chemotactic response increased.

The influence of zinc on the immune system and on physiological reactions is also dependent on the chemical source of zinc. Organic chelated zinc (ZnMethionine) is more available to the fish than inorganic zinc (ZnSO_4) (Lim et al. 1996; Paripatananont and Lovell, 1995). For example, three to six times more zinc was required for maximal weight gain and bone zinc deposition when ZnS was fed instead of ZnMet, and ZnMet was more potent than ZnS for antibody production and in protecting channel catfish against *E. ictaluri* (Paripatananont and Lovell, 1995).

Fish can absorb zinc from both water and dietary sources. However, dietary zinc is more efficiently absorbed than waterborne zinc (Lim et al. 2001). Zinc availability from dietary sources is also influenced by the presence of other compounds such as phytic acid and calcium. The amount of inorganic zinc required by channel catfish for normal growth and bone zinc deposition has been estimated to be 20 mg/ kg in an egg-white based diet (Gatlin and Wilson, 1983), and over 100 mg/kg in a practical diet containing phytic acid (Gatlin and Wilson, 1984). Moreover, the presence of high calcium increases the zinc requirement because calcium promotes the complexing of zinc with phytate (Lim et al. 2001). In fish, zinc deficiency can cause growth depression, lens cataracts, short body

dwarfism, poor growth, loss of appetite, high mortality, skin and fin erosion (Lim et al. 2001; Ogino and Yang, 1978, 1979).

7.9. Nucleotides

The nucleotides consist of a nitrogen base (pyrimidine and purine), a sugar (deoxyribose in DNA and ribose in RNA), and one or more phosphate groups. Nucleosides lack the phosphate group. Ingested nucleotides are hydrolyzed in the intestinal lumen into nucleosides, and then are transported inside the enterocytes where they are directly used for DNA or RNA synthesis or are degraded. Part of the nucleosides is transported inside the portal system to the liver where they are utilized or degraded.

Nucleotides are provided endogenously by de novo synthesis in tissues, such as the liver. However, this process is very energy-consuming and so cells utilize preferentially exogenous sources of nucleotides, such as from the diet (Uauy, 1994). Although most tissues can synthesize nucleotides de novo; other cells, including immune cells and intestinal cells, lack this ability and must depend on pre-formed nucleotides (Quan, 1992). In medical therapy, free nucleotides are added to patient diets to help speed recovery from disease. Dietary nucleotides promote growth, maturation of developing gut, regeneration, and healing of intestine mucosa (McCauley et al. 1998). Dietary nucleotides can stimulate the immune system, and consequently, nucleotide-deficient diets are used in organ transplant protocols (Van Buren et al. 1994). Mice fed nucleotide-supplemented diets had lower mortality than mice fed nucleotide-free diets after challenge with *Staphylococcus aureus* (Kulkarni et al. 1986) or *Candida albicans* (Carver, 1994). This increase in resistance to infection is reported to be a consequence of increased phagocytic activity of murine peritoneal macrophages (Kulkarni et al. 1986),

increased T-cell dependent antibody production (Jyonouchi, 1994), increased natural killer cell (NK) activity (Carver, 1994), enhanced interleukin-2 production (Carver, 1994), and elevated bone marrow cell and peripheral neutrophil numbers (Matsumoto et al. 1995).

Few studies have investigated the effect of dietary nucleotides in fish. Dietary nucleotides increased resistance of rainbow trout challenged with *Vibrio anguillarum*, of Atlantic salmon challenged with Infectious Salmon Anaemia Virus (ISAV), of coho salmon challenged with *Piscirickettsia salmonis* (Burrells et al. 2001), and of Atlantic salmon challenged with *Aeromonas salmonicida* (Burrells, unpublished data). Most recent studies have shown that intracoelomic administration of nucleosides may give better results than the use of a nucleotide supplemented diets (Veerabagu et al. 1996), because some of the nucleotides administered orally are degraded in the intestinal tract.

7.10. Beta-Glucans

Beta-1-3- and beta-1-6-glucans are derived from yeast and fungi. They are among the most ancient microbial cell wall components and for this reason higher organisms may have developed recognition mechanisms. The most commonly used beta-glucans are derived from the inner cell wall of baker's yeast, *Saccharomyces cerevisiae*. Brewery, torula yeast, fungi, and algae are also sources of beta-glucans currently in use. The beta-1-3-glucan layer is covered by mannose-rich proteins and by lipids that can be removed by trypsin digestion and boiling. Differences in the immunostimulatory effect of glucans have been attributed to differences in their source, isolation procedures, quantity, length, and relative proportion of beta-1-3- to beta-1-6- linked side chains (Raa, 1996).

Glucans can be added to the diet, can be administrated by injection or immersion, and can be used as vaccine adjuvants.

Glucans can activate macrophages through specific receptors (Engstad and Robertsen, 1993) stimulating an increase of the respiratory burst and of phagocytic activity. Atlantic salmon macrophages tested *in vitro* with *Aeromonas salmonicida* showed an increased phagocytic activity against sheep red blood cells after 3 days and an increase in respiratory burst activity after 4-7 days. The respiratory burst data also indicate that high concentrations of beta-glucans ($> 10 \mu\text{g/mL}$) may induce the production of self-inhibitory compounds in the macrophages or may be toxic for them (Jorgensen, J. B. and Robertsen B., unpublished data).

Mammalian macrophages produce cytokines that may activate lymphocytes (IL-1) or themselves (tumor necrosis factor alpha). IL-1 activates both the specific and nonspecific defense systems. Both yeast glucan and lentinan elicit the production of IL-1 in mammalian mononuclear phagocytes (Abel and Czop, 1992). IL-1 like molecules have been demonstrated in several teleosts (Secombes et al. 2001). Tumor necrosis factor (TNF) may also be involved in the activation of Atlantic salmon macrophages by yeast beta-glucan. Polyclonal rabbit antiserum against human recombinant TNF was able to reduce the respiratory burst activity of isolated Atlantic salmon macrophages activated by yeast glucan. However, normal rabbit serum and rabbit antiserum against human IL-1 did not reduce the glucan-induced respiratory burst activity of the macrophages (Jorgensen, J. B. and Robertsen B., unpublished data). The migration of leukocytes may be mediated by chemoattractants that in part are generated by glucan-mediated activation of the

complement (C5a) and in part by eicosanoids (leukotriene B4 and lipoxin A4) released from macrophages (Secombes and Fletcher, 1992).

Glucans can increase the survival of fish to pathogens. In Atlantic salmon vaccinated against *A. salmonicida*, supplementation of beta-glucan induced a higher protection and an enhancement of the level of antibody against bacterial membrane proteins, but not against lipopolysaccharides. These data suggest that glucans have no effect on B-cells but may stimulate T-cell function or antigen presentation of bacterial surface proteins (Nikl et al. 1993; Rorstad et al. 1993). In turbot vaccinated against *Enterococcus* sp., glucans induced an enhancement of the non-specific defense mechanism (Toranzo et al. 1995). Similar results were observed in yellowtail challenged with *Enterococcus seriolicida* (Itami et al. 1996). Reduced mortality to *A. hydrophila* or *Pseudomonas fluorescens* challenge was observed in swordtails, rosy barbs, and black tetras fed glucans (Tuernau et al. 2000). Glucans from lentinan, schizophyllan, and scleroglucan increased survival rate of carp challenged with *Edwardsiella tarda* (Yano et al. 1991). Glucans enhanced nonspecific immune responses, but not resistance to *E. ictaluri* in catfish (Duncan and Klesius, 1996), and to *Vibrio anguillarum* in turbot (De Baulny et al. 1996).

7.11. Spirulina

Recent studies have begun to focus on the use of Spirulina as an immunostimulatory compound. Spirulina is a blue-green alga and its immunostimulatory action appears to be due to the presence of a polymerized sugar molecule, Calcium-Spirulan. This molecule contains both sulfur and calcium, and is composed of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, and glucuronic acid.

Spirulina has a content of 62% amino acid, and it is rich in vitamin B12, carotene, and xanthophylls. Its soft cell wall is made of a complex of sugars and protein, and it is easily digested.

Administration of Spirulina in diets helps to inhibit viral replication, strengthens both cellular and humoral arms of the immune system, and causes regression and inhibition of cancers (Hayashi et al. 1993; Hayashi and Morita 1996; Patterson, 1993). In the presence of Spirulina extract, it appears that viruses can not penetrate the cell membrane, and therefore become unable to infect cells. These viruses are then eliminated by the body's natural defenses. Several studies (Hayashi et al. 1993; Hayashi and Morita 1996; Patterson, 1993) have demonstrated that Spirulina inhibits replication of several enveloped viruses including Herpes simplex virus type 1, human cytomegalovirus, measles virus, mumps virus, influenza A virus and HIV-1. Spirulina enhances cell nucleus enzyme activity and the DNA repair system. It also enhances the body's ability to generate new blood cells, especially in spleen and thymus. Spirulina causes increases in macrophage number and phagocytic activity. Spirulina can also accelerate the production of antibodies and cytokines (Hayashi et al. 1994; Qureshi et al. 1995; Pang et al. 1988; Zhang et al. 1994).

7.12. Other Immunostimulants

Immunostimulant activity has been attributed to several other compounds such as chitin, k-carrageenan, lipid, gossypol, and levamisole (Anderson, 1992; Barros et al. 2000; 2002; Fracalossi and Lovell, 1994; Raa, 1996). Significant improvement in agglutinating antibody titers and mean macrophage migration in the presence of exoantigen was enhanced with increasing levels of dietary cotton seed meal (Barros et al.

2000; 2002). Replacement of soy bean meal with cotton seed meal significantly reduced cumulative mortality of catfish challenged with *Edwardsiella ictaluri*. Gossypol present in cotton seed meal could be one of the compounds responsible for these enhancements. Gossypol has been shown to immobilize and lyse African trypanosomes (*Trypanosoma brucei brucei*) *in vitro* interfering with glycolysis in the parasite (Eid et al. 1988). It has also been shown to be effective against two other parasites, *Trypanosoma cruzi*, the cause of Chagas's disease, and *Plasmodium falciparum*, the cause of malaria (Eid et al. 1988). Polsky et al. (1989) also reported that gossypol inactivated human immunodeficiency virus in an *in vitro* system.

Levamisole is a drug used to treat helminth parasites. It also acts as an immunostimulant. One problem with use of levamisole is that the dosage range that produces a stimulatory response may be rather narrow. Higher doses may cause immunosuppression and interfere with vaccines in an unpredictable or even negative way (Anderson, 1992).

CHAPTER 8

DESIGNING A BIOSECURITY LABORATORY AND RECIRCULATING SYSTEMS FOR CHALLENGE AND VACCINE EXPERIMENTS WITH SMALL FISH

8.1. Introduction

Aquaculture is a growing industry and with intensive fish farming there is the need to develop drugs and vaccines for many important diseases and species of fish. Unfortunately many research facilities are not equipped with a biosecure laboratory. In our research we have developed challenge models and vaccination efficacy tests for ornamental fish and for this purpose we designed a biosecure laboratory with recirculating systems that can be easily constructed. This facility and systems have been developed for small fish, but with a few modifications, this design can be built to suit studies conducted with larger fish.

Biosecurity and disease contained environments are the key characteristics of this type of laboratory and should be present in every aquaculture facility. Biosecurity refers to measures taken to prevent or reduce the risk of transmission of pathogens to healthy animal populations and should be an integral part of daily management of aquaculture facilities. Disease containment refers to specific measures that are taken following a disease outbreak in a farm and also involve procedures to follow after the diseases have been eliminated. Important components in biosecurity and disease containment are quarantine, sanitation, and disinfection. Quarantine is a period of observation of new imported stocks of fish or treated fish to ensure their healthy state. Usually this period is 40 days long (quarantine comes from the Italian word *quaranta* that means forty), while

sanitation includes a series of procedures, such as elimination of uneaten food, organic waste, and dead fish, to maintain a clean environment; disinfection refers to a series of procedures to reduce the spread of pathogens using chemicals, ultraviolet light, or ozone (Yanong, 2003).

One of the main purposes of biosecurity is to avoid the entry of pathogens into the facilities, even if some pathogens are normally found in the environment and normally are considered less of a concern (Yanong, 2003). It is important to consider all the possible routes of transmission of pathogens which can be direct (contact between infected and susceptible animals), indirect (by nets or other equipments, boots, human, insects or other animals), horizontal (direct or indirect contact), or vertical (via gametes). Measures to eliminate pathogens vary and depend on the type of facility, known and potential disease vectors, possible diseases, and farm management practices. For example, closed recirculating systems have a higher biosecurity level than open systems. Common biosecurity measures include stocking specific pathogen free fish or of a known health status (Moss and Argue, 2001), separation of different groups of fish, correct stocking density, sanitation of equipment and facilities before restocking, controlled access of people, prevention of direct or indirect contact with other farms, proper waste management, good quality feed and proper storage, water sterilization through UV lights, good water quality, efficient filtration systems, and choice of the right disinfectants. The choice of disinfectants is very important because not all chemicals are effective against viral, bacterial and fungal disease and, in addition, disinfectants must cause no harm to the environment and to people. Cost effectiveness and the real possibility of application are among the main considerations in developing a biosecurity plan. Biosecurity is part of

any good management plan. Many biosecurity measures are common sense practices and require little expense, but commitment of all personnel.

For our research we have converted a concrete storage room into a biosecurity laboratory. It is important to use a concrete building material because it is more resistant and the walls can not be easily crossed by insects or small animals that could act as vectors for pathogens. It is also important to build the biosecure laboratory higher than ground level to avoid any flooding due to heavy rains.

8.2. Laboratory Design

For our research a 4 x 4 m concrete room containing two recirculating systems was converted into a biosecure laboratory (Figure 8-1). To decrease the risk of carrying or spreading pathogens, two layers of plastic curtains were hung behind the entrance door, a footbath with benzalkonium chloride, 70% isopropyl alcohol spray bottles for hand washing, gloves and other coverings garment were also used. During the studies, only plastic boots were worn in the laboratory. A safety rule we always followed was to not enter any fish facilities on the farm after working in the laboratory. The room was equipped with a heat pump with cooling and heating capabilities to maintain a constant temperature of 25-27°C.

Due to the absence of a water supply system, well and reverse osmosis water was brought inside the laboratory through a removable hose connected to the external wall, and stored in four 400 L tanks (Figure 8-2). Water was then aerated to eliminate any possible hydrogen sulfide and carbon dioxide present and to increase the oxygen content. A submersible pump was used to transfer the water from these tanks to the recirculating systems, and from there another submersible pump located in the sump tank was used to

dump water to a 7500 L reservoir tank located outside the laboratory. A ball valve was inserted in the outgoing line from the laboratory to avoid the passage of small animals or insects. Before dumping the water from the recirculating systems, chlorine was added in the outside reservoir tank to reach a concentration of 150 mg/L, and after 3 days the water was flushed. Due to the lack of water lines in the room, a 400 L tank was used as a sink, and was connected to the incoming water line by a garden hose.

8.3. Recirculating Systems Design

In order to run two different experiments at the same time, two independent recirculating systems were constructed inside the biosecure laboratory (Figure 8-1 and 8-3). Each system was composed of 25 tanks of 38-L volume and equipped with a bead filter which acted as both a biological and a mechanical filter, and a UV sterilization unit. To ensure that each tank received water at the same pressure, a 1/8 horsepower pump was used to send water from a bottom sump to a sump located higher than the other tanks, and from there the water reached each single tank by gravity. The water flow in each tank was regulated via a ball valve. For these studies, the filter design was more than sufficient for removing particulate waste. If a more efficient mechanical filtration is needed, aside from the bead filter, several mechanical filtration materials can be layered perpendicular to the water flow and inserted in the bottom sump.

8.4. Sterilization and Start up Protocols

At the beginning of each experiment, commercial mixes of biofilter bacteria (BioSpira®, Marineland, Moorpark, California) were used to inoculate the bead filters. Ammonia (Clear Sea Mist Ammonia, Laundry Aids Inc. Carlstadt, New York) was gradually added daily to stimulate the growth of the ammonia and nitrite consuming

bacteria. When the bacterial population could metabolize 100 mL ammonia per day, the same calculated amount of ammonia produced by the biomass of fish of stocked fish, the systems were considered ready to be stocked with fish. In order to speed up this process, which can take up to six to eight weeks, two bead bioreactors were built (Figure 8-4). Each bead bioreactor was comprised of a rectangular 400 L tank. The water was kept in motion by air bubbles flowing out from pvc pipes forming a “8” shape located at the bottom of the tank. After sterilization, the bead filters in the recirculating systems were filled with beads from the bioreactors. This procedure reduced the start up time to 7-10 days.

At the end of each experiment the recirculating systems and the room were sterilized to be sure that new fish were not exposed to specific bacteria. Chlorine (Clorox, 6% sodium hypochlorite, The Loro Company, Oakland, CA) was added to the systems for 24 h at a concentration of 150 ppm, and straight chlorine was poured on the floor and sprayed on equipment.

8.5 Conclusions

Our room and system design demonstrate that it is possible to easily build an economically biosecure laboratory for research purposes. Studies completed in this laboratory demonstrate the utilitarian success of our design. The biosecurity of the room is supported by the fact that even though this laboratory is located a few meters next to several greenhouses that we used for other research purpose, in 3 years of research conducted in the area, no disease outbreak connected with our research (i.e., streptococcal infections) has been observed. Research dealing with viral diseases will need higher biosecurity standards, but our laboratory design can be used as a starting point.



Figure 8-1. View of the biosecurity room. The two independent recirculating systems are located at the side of the room. In the middle of the room there is the tank used as a table-sink.



Figure 8-2. Close-up view of one of the recirculating systems.



Figure 8-3. View of three of the four 400 L tanks used to store incoming water. Visible on the left is one of the plastic curtains used to avoid the spread of pathogens (bacteria). The curtain was located between the recirculating systems and the water storage tank to avoid the contamination of the new stored water.

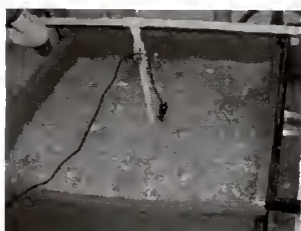


Figure 8-4. View of one of the beads bioreactors. The white mass in the water are beads kept in movement by air blown by air pump.

CHAPTER 9
DEVELOPING CHALLENGE MODELS AGAINST *Streptococcus iniae* IN RED-TAIL
BLACK SHARK (*Epalzeorhynchus bicolor*) AND RAINBOW SHARK (*E. erythrurus*),
FAM. CYPRINIDAE

9.1. Introduction

Streptococcosis is a very serious disease for farm fish and may cause up to 75% mortality in closed culture systems (Perera et al. 1994) and up to 50% in ponds (Eldar et al. 1997). Streptococcosis is one of the main diseases in yellowtail in Japan (Bang and Sung-Hee, 1993) and in tilapia and rainbow trout in Israel (Bercovier et al. 1997). Streptococcal diseases have been reported in many species of marine and freshwater fish (Aoki et al. 1990; Bromage et al. 1999; Bunch and Bejerano, 1997; Colorni et al. 2002; Eldar et al. 1995a; 1999; Ferguson et al. 1994; Ikeda and Minami, 1982; Kitao et al. 1981; Kusuda et al. 1978; Lio-Po et al. 1998; Park et al. 1987; Ming-Chen et al. 1985; Perera et al. 1997; 1998; Romalde et al. 2000; Sako, 1998; Shoemaker et al. 2001; Stoffregen et al. 1996; Ugajin, 1981) and both in farm and wild fish (Baya et al. 1990; Colorni et al. 2002; Zlotkin et al. 1998). Several studies have been conducted to develop a vaccine against this group of bacteria in yellowtail (Iida et al. 1982; Kusuda and Takagi, 1983), tilapia (Bercovier et al. 1997; Eldar et al. 1995b; Klesius et al. 2000; Klesius et al. 2001), and rainbow trout (Eldar et al. 1997; Sakai et al. 1987). Also an efficient combined vaccine against *Streptococcus* sp. and *Vibrio anguillarum* has been developed (Sakai et al. 1995).

One of the first steps in fish vaccine research is to develop a challenge model, that is, to determine the lethal dose of bacteria needed to kill a predetermined percentage of

fish in a fixed period of time. In most studies the lethal dose of bacteria should kill 70% of the fish population in 14 days; however these numbers may vary among research groups (Kimura and Kusuda, 1979; Nordmo and Ramstad, 1997; Perera et al. 1997; Sako, 1998). Fish can be infected by immersion for a few seconds or minutes in a tank with a chosen bacteria concentration, or by the introduction of sick fish, or by intracoelomic or intramuscular injection of bacterial cultures. In our research, fish were infected by intracoelomic injection because this is a more replicable technique among experiments than the other challenge routes, and is generally more efficient in establishing an infection (Kimura and Kusuda, 1979; Perera et al. 1997; Sako, 1998). Nevertheless, in some research no differences were observed at the end of the experiment in vaccinated fish between the intracoelomic and cohabitation challenge methods (Nordmo and Ramstad, 1997).

In this study red-tail black shark (RTB shark) and rainbow shark (family: Cyprinidae) were chosen as target species because they are economically important to the Florida ornamental fish industry and are very susceptible to streptococcosis. This research is the first step to develop a vaccine against *Streptococcus iniae* for these ornamental fish, and the goal was to find the concentration of *S. iniae* that would cause 70-80% mortality of RTB and rainbow sharks.

9.2. Materials and Methods

Experimental animals and systems. The research was conducted at the Tropical Aquaculture Laboratory, University of Florida (Ruskin, FL) using two recirculating systems with 25 tanks of 38-L volume. RTB shark (mean weight 1.3 ± 0.4 g and TL 5.2 ± 0.5 cm) and rainbow shark (mean weight 1.0 ± 0.2 g and TL 4.9 ± 0.4 cm) juveniles

were obtained from an ornamental fish farm in Hillsborough county (FL) where they were raised in ponds. A representative group of 30 individuals for species was measured at the beginning of the research. To verify the *S. iniae*-free status of the fish, brain and kidney tissues were sampled from 5 representative selected fish before each experiment. The samples were spread on Tryptic Soy Agar with 5% sheep's blood (Physician's Laboratory Supply, Troy, Michigan) and incubated at 37°C for 48 h.

Temperature, ammonia, and nitrite in the systems were measured daily; alkalinity, hardness and pH once at week. The water in the system was maintained at $25 \pm 1^\circ\text{C}$. Ammonia and nitrite concentrations were always close to 0 mg/l. Alkalinity and hardness were 110 ± 10 mg/L and 115 ± 15 mg/L respectively; pH was 7.5.

Bacterial strain. The strain of *S. iniae* used in this research was isolated from infected rainbow sharks submitted to our laboratory and belonging to the same ornamental fish farm from in the Tampa area that provided the sharks for these experiments. After necropsy and bacterial culture, *S. iniae* infection has been determined to be the cause of the disease. Bacterial ID was obtained with the BIOLOG MicroLog3 version 4.00 System (Biolog, Inc., Hayward, California) and with standard microbiological tests (Table 9-1). Single representative colonies from the original plates were utilized to prepare a stock broth culture. The bacteria were first purified by subculture in BBL TSA II (Physician's Laboratory Supply, Troy, Michigan) at 37°C for 24 h. A few of the resultant purified colonies were grown for 18-24 h at 37°C in two 250 mL brain heart infusion (BHI) broth (Physician's Laboratory Supply, Troy, Michigan) flasks enriched with sterile bovine serum to 1% v/v (Fisher Scientific, Pittsburg, Pennsylvania). For calculating the bacterial concentration, 1 mL BHI broth culture was

used for preparing serial dilutions in 9 mL saline solution; successively 1 mL of each dilution solution was spread in a BBL TSA II agar plate and cultured at 37°C for 24 h. After incubation the average culture count of the BHI broth was 3×10^7 bacteria/mL. The broth cultures were then mixed 1:1 with sterile evaporated skim milk and transferred to 250 cryovials of 2.0 mL volume. All the aliquots were immediately frozen and stored at -70°C.

For each experiment, a *S. iniae* culture was prepared using one frozen cryovial. The cryovial was first defrosted at room temperature, and then used to prepare a 250 mL brain heart infusion (BHI) broth (Physician's Laboratory Supply, Troy, Michigan). The broth was grown for 18-24 h at 37°C. The bacterial concentration was calculated using the protocol described previously. Sterile BHI broth was used to prepared dilution of the bacteria culture.

Experimental design. The challenge model for RTB shark was based on the results of three experiments (experiments 1-3), and then to check this model in rainbow shark two other experiments were performed (experiments 4 and 5). In experiment 1 RTB shark were injected with concentrations of *S. iniae* between 1.5×10^7 and 1.5×10^{10} (Table 9-2). These concentrations were chosen based on vaccine experiments against *S. iniae* run on other species of fish found in the literature (Eldar et al. 1997; Hortvitz et al. 1997; Iida et al. 1982; Kimura and Kusuda, 1979). Because this range of concentrations caused 100% mortality in two days, it was decided to run a second experiment using a much lower range of bacterial concentration (between 1.5×10^1 and 1.5×10^4). Experiment 3 was run to verify the results of the second experiment. To test the validity of the challenge model obtained for RTB shark in rainbow shark, experiment 4 was run

injecting concentrations of *S. iniae* between 1.5×10^3 and 1.5×10^6 (Table 9-3). A fifth experiment with rainbow shark was run to verify the results of the previous experiment.

All five experiments used the following protocol. Three tanks were randomly assigned for each treatment and for the control group. Each tank was stocked with 25 fish. After a week of acclimation, fish were challenged by an intracoelomic injection of 0.05 mL *S. iniae* culture. The control group was injected with sterile BHI. In tables 9-2 and 9-3 are reported the bacterial dose injected for each experiment. For injection of *S. iniae* doses higher than 10^7 bacteria/mL, bacterial cultures were first centrifuged at 2000 IPM for 30 minutes and then the pellet was resuspended in sterile BHI to reach the right bacterial concentration. Fish mortality was recorded for 14 days after challenge. During this period, dead fish were removed 2 times per day, only in the experiment 1 dead fish were removed 3 times at day. In each experiment, representative brain and kidney cultures were taken on 70-80% of dead fish for each treatment to verify the cause of mortality. *Streptococcus iniae* was identified from the bacteria cultures by (1) observation of the colony morphology in the bacteria cultures, (2) observation under light microscope with 100x or 400x magnification of 50% of representative chosen bacteria cultures, and (3) by gram stain of 30% of representative chosen bacterial cultures. For each trial, 12 moribund fish were randomly chosen and preserved in 10% formalin. Histological slides were prepared using 10 randomly fish selected from all preserved fish.

Statistical analysis. ANOVA analysis and Tukey's post hoc test were run for each experiment using the statistical program SPSS 12.0 (SPSS Inc., Chicago, IL). An asin(square root) transformation was performed on the mortality data expressed as

percentage. The results of the ANOVA analyses were considered significant at a $p\text{-value} < 0.01$; then the Tukey's test was considered at an $\alpha = 0.05$.

9.3. Results

Darkening and lethargy were the first signs observed in challenged RTB and rainbow sharks, followed by the classical signs of streptococcosis. Hemorrhages could be observed on the ventral side of the body and on the head, and at the base of the pelvic and pectoral fins. Exophthalmia was observed in a low percentage of fish (~10%). Moribund fish demonstrated the characteristic spinning swimming pattern. Histological observation revealed leukocyte infiltration in the intestinal area, spleen, posterior kidney and brain. Necrosis and tissue degeneration were observed in the same organs in addition to degeneration of the renal tubules. No granulomas were observed in tissues. *Streptococcus iniae* was recovered from all cultured moribund or dead sharks and recognized by the white pin point shape of the bacterial cultures on BBL TSAII agar, by the observation of cocci with the light microscope from samples taken from the bacteria cultures, and by positive results of the gram stains.

All concentrations of *S. iniae* tested in the first experiment caused 100% mortality of RTB shark in the first two days after bacteria challenge (Table 9-2 and Figure 9-1). Less than twelve h after the bacteria injection, the fish began to show classical signs of streptococcosis and the first mortalities were recorded.

In the second experiment (Table 9-2 and Figure 9-2) average mortalities of $44 \pm 13\%$ and $65 \pm 18\%$ after 14 days from bacteria challenge were observed when fish were injected respectively with 1.5×10^3 and 1.5×10^4 bacteria. Challenge with lower doses did not cause significantly higher mortality than in the control group.

In the third experiment (Table 9-2 and Figure 9-3) average mortalities of $66 \pm 17\%$, $75 \pm 11\%$ and $86 \pm 5\%$ after 14 days from bacterial challenge were observed when fish were injected respectively with 1.5×10^3 , 10^4 and 10^4 bacteria. Also in this trial challenge with lower doses did not cause significantly higher mortality than in the control group.

Results of experiments 2 and 3 showed that injection of 1.5×10^4 bacteria/fish constantly caused mortality to be close to 70% in 14 days. Injection of 1.5×10^3 bacteria/fish caused lower mortality, and the mortality rate was less consistent among trials. The injection of 1.5×10^5 bacteria/fish caused mortality exceeding the prefixed chosen target of 70%. For this reason injection of 1.5×10^4 bacteria/fish was chosen as the best dose to cause mortality to be close to 70%. In RTB shark no mortalities were observed after 11-12 days.

The results of experiments 4 - 5 with rainbow shark (Table 9-3, Figure 9-4 and 8-5) showed that injections of 1.5×10^4 bacteria/fish resulted in an average percentage mortality close to 80% in 14 days, injection of 1.5×10^3 bacteria caused a lower mortality and less consistently among trials, and injection of higher doses caused 100% mortality. These results confirmed the challenge model obtained with RTB shark, although there were a few differences in mortality rate. For example, when compared to that of RTB shark, rainbow shark mortality stopped after 3 - 4 days, except for one treatment (Figure 9-4).

9.4. Discussion

Darkening and lethargy were the first signs observed in infected RTB and rainbow sharks. Moribund and dead fish presented hemorrhages on ventral side of the

body, of the head, at the base of the pelvic and pectoral fins, and exophthalmia was observed on a low percentage of fish (~10%). These are classic signs of streptococcosis, as observed in tilapia, hybrid bass and ornamental fishes (Ferguson et al. 1994; Liu et al. 1990; Perera et al. 1998) where the *S. iniae* infection was characterized by exophthalmia, corneal opacity, skin lesions, pronounced congestion and hemorrhages around the base of the pectoral fins, in correspondence of the heart, and in the eye.

Histological examination of RTB and rainbow sharks revealed leukocyte infiltration in the intestinal area, spleen, posterior kidney and brain. Necrosis and tissue degeneration was also observed in these organs in addition to degeneration of the renal tubules. These observations are in accordance with the pathological outcomes of *S. iniae* infections in which brain, spleen and kidney; but also the pericardial area, liver and intestine can be affected (Camus, 2002; Eldar et al. 1995a; Ferguson et al. 1994; Ikeda and Minami, 1982; Kusuda and Kimura, 1978; Kusuda and Kawai, 1982; Liu et al. 1990; Neely et al. 2002; Perera et al. 1998). Spleen and posterior kidney may have major congestion, pronounced degeneration and necrosis due to the high bacterial trapping capability of these organs. Blood vessels and capillaries may have occasional thrombosis due to an elevated number of bacteria in the blood. The observed leukocyte infiltration is caused by a strong inflammatory response of the host that causes acute necrotizing reactions and systemic neutrophil and macrophage exudation in several organs and tissues (Ferguson et al. 1994; Neely et al. 2002).

Moribund fish showed a “spinning” swimming that is a common presentation of *S. iniae* infections and it might be correlated with the massive cellular infiltration in the brain associated in some cases with meningeal granulomas (Ferguson et al. 1994; Perera

et al. 1998). However, in our case we never observed such granulomas. *Streptococcus iniae*, similar to other streptococci belonging to group B (GBS), have a tropism for the brain, and this tropism is one of the main causes of death. The invasion rate of *S. iniae* into different organs has been carefully observed and described in yellowtail (Kusuda and Kawai, 1982; Kusuda and Kimura, 1978; Sako, 1998) where in the first two days after bacterial challenge, there was an increase in bacterial count in blood, spleen, kidney and brain. Afterward, the bacterial count in the blood, spleen and kidney decreased or remained constant, while in the brain it increased until mortality onset. The exact mechanism used by *S. iniae* to cross the blood-brain barrier (BBB) is still not well understood, but it involved the production of the cytolysin streptolysin S (SLS) that is required for local tissue necrosis (Camus, 2002; Fuller et al. 2002; Kimura and Kusuda, 1979; Kusuda and Hamaguchi, 1989; Romalde et al. 2000; Sakai et al. 1992). Perhaps *S. iniae*, like other GBS, may elicit its own endocytotic uptake from the microvascular endothelial cells and pass the BBB (Nizet and Rubens, 2000). Otherwise, as observed with *S. suis*, *S. iniae* may adhere and damage the microvascular endothelial cells through the action of SLS, and then proceed from the circulation to the central nervous system (Charland et al. 2000). The subsequent bacterial replication within the CNS may provoke an overwhelming host inflammatory response that in combination with the disruption of the BBB could be fatal (Doran et al. 2003). *Streptococcus iniae*, like other GBS, could also secrete other factors that break down the extracellular matrix helping to spread the bacteria, or that inactivate inflammatory mediators, such as C5a, thus decreasing the recruitment and opsonophagocytic capacity of neutrophils (Cheng et al. 2002; Liu and Nizet, 2004).

From the results of the experiments with RTB shark, it is possible to design a very plausible challenge model against *S. iniae* by the intracoelomic injection route in 5 cm RTB shark kept at 25°C. In this model consistent mortality close to 70% in 14 days after bacterial challenge can be obtained by injecting fish with 1.5×10^4 bacteria. The fish began to show the first signs of streptococcosis and mortality after less than one day post-challenge. Mortality due to *S. iniae* was confirmed by the positive results of the brain and kidney cultures of moribund and dead fish.

Experiments 2 and 3 were conducted in different seasons and using different stocks of juvenile RTB shark, for this reason the differences in mortality rates observed between the two experiments might also be correlated to genetic variation or environmental factors that influenced the fish when they were raised in ponds (i.e. water temperature, stocking density, feed consumption, dissolved oxygen). Variations in mortality rates among trials are not uncommon, as observed in studies on infection of *S. iniae* in yellowtail, for example (Sako, 1998).

The results of the experiments with rainbow shark demonstrate the validity of the challenge model developed for RTB shark also for this species, even though there were some small differences. The injection of 1.5×10^4 bacteria/fish caused a higher mortality rate in rainbow than in RTB shark, and in rainbow shark mortalities stopped 3–4 days after challenge, and not in 11–12 days as in RTB shark. These observations suggest that rainbow shark may be more susceptible to *S. iniae* than RTB shark, as the results of our experiments on vaccine efficiency against *S. iniae* in sharks (Chapter 12) also seem to show.

From the results of several *S. iniae* challenges by injection in other fish, it appears that sharks are as susceptible to *S. iniae* as zebrafish (Neely et al. 2002), but more susceptible than carp (unpublished data), tilapia (Hortvitz et al. 1997), rainbow trout (Eldar et al. 1997), and yellowtail (Iida et al. 1982; Kimura and Kusuda, 1979). However differences could also be caused by variation in fish size, age, water temperature and other environment factors.

9.5. Conclusions

The results of this research have been useful for creating a challenge model against *S. iniae* for use in RTB and rainbow shark under the conditions of this research. Some differences in bacterial susceptibility and in the progression of the infection were observed between the two species. The results of this experiment will be used to design trials to test vaccine efficiency against *S. iniae* in these two species of fish.

Table 9-1. Characteristic of *S. iniae* isolated from diseased rainbow shark together with data on the type of strains isolated from other species of fish.

Test	Present study Rainbow shark	Collins and Lewis (1994) Tilapia hybrid	Sako (1998) Yellowtail, ayu, common mackerel, Japanese flounder
Cell morphology	spherical, long chains	spherical	spherical
Gram stain	+	+	+
Growth at 10°C	+	+	ND
Growth at 45°C	-	+	ND
Growth in 6.5% NaCl	-	-	ND
Catalase	-	-	-
Hemolysis	alpha/beta	beta	beta
Hydrogen sulfide production	-	ND	-
Esculin hydrolysis	-	ND	+
Hippurate hydrolysis	-	-	-
Voges-Proskauer	-	-	-
Acid from:			
Arabinose	+	-	-
Glucose	+	+	+
Lactose	-	-	-
Mannitol	-	+	+
Raffinose	-	-	-
Salacin	-	-	+
Sorbitol	-	-	-
Trehalose	+	+	+
D-xylose	-	-	-

Table 9-2. Average cumulative mortality (%) 14 days after bacterial challenge for the experiments with RTB shark, 3 replicates were used per treatment and each tank was stocked with 25 fish.

Specie	Trial n	Treatment bacteria/fish	Final Mortality %±SD	Significance
RTB shark (<i>E. bicolor</i>)	1	Control	0±0	a
		1.5x 10 ⁷	100±0	b
		1.5x 10 ⁸	100±0	b
		1.5x 10 ⁹	100±0	b
		1.5x 10 ¹⁰	100±0	b
	2	Control	0±0	c
		1.5x 10 ¹	5±0	c
		1.5x 10 ²	13±6	c,d
		1.5x 10 ³	44±13	d,e
		1.5x 10 ⁴	65±18	e
	3	Control	0±0	f
		1.5x 10 ¹	7±9	f
		1.5x 10 ²	3±5	f
		1.5x 10 ³	66±17	g
		1.5x 10 ⁴	75±12	g,h
		1.5x 10 ⁵	86±5	h,i

In the treatment column the concentration of injected *S. iniae* (bacteria/mL) used for each trial are reported, and in the last column are reported the results of the Tukey's test. ($p_2 = 0.0017$, $p_3 = 3.6 \times 10^{-8}$, $\alpha = 0.05$).

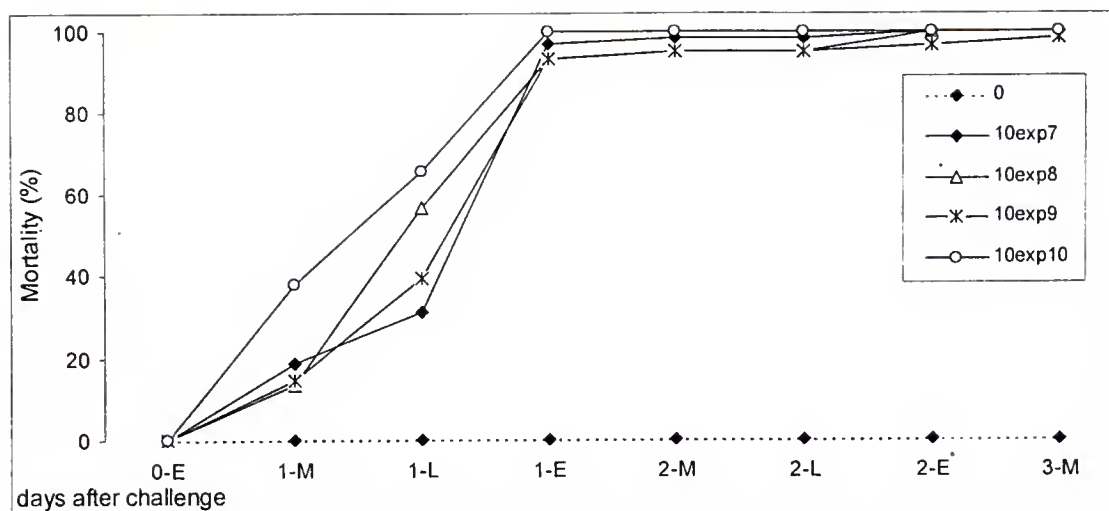


Figure 9-1. Average mortality rates (%) observed in experiment 1 with RTB shark. Number in abscissa refers to day after the challenge (day 0). M= morning (08:00), L= lunch (14:00), E= evening (21:00); 3 replicates were used per treatment and each tank was stocked with 25 fish.

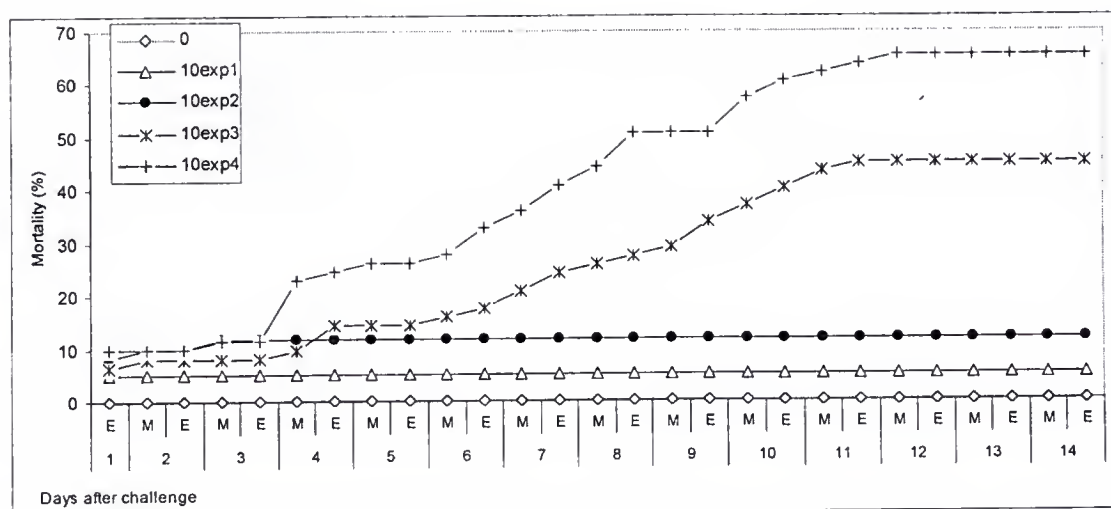


Figure 9-2. Average mortality rates (%) observed in experiment 2 with RTB shark. Number in abscissa refers to day after the challenge (day 0). M= morning (09:00), E= evening (21:00); 3 replicates were used per treatment and each tank was stocked with 25 fish.

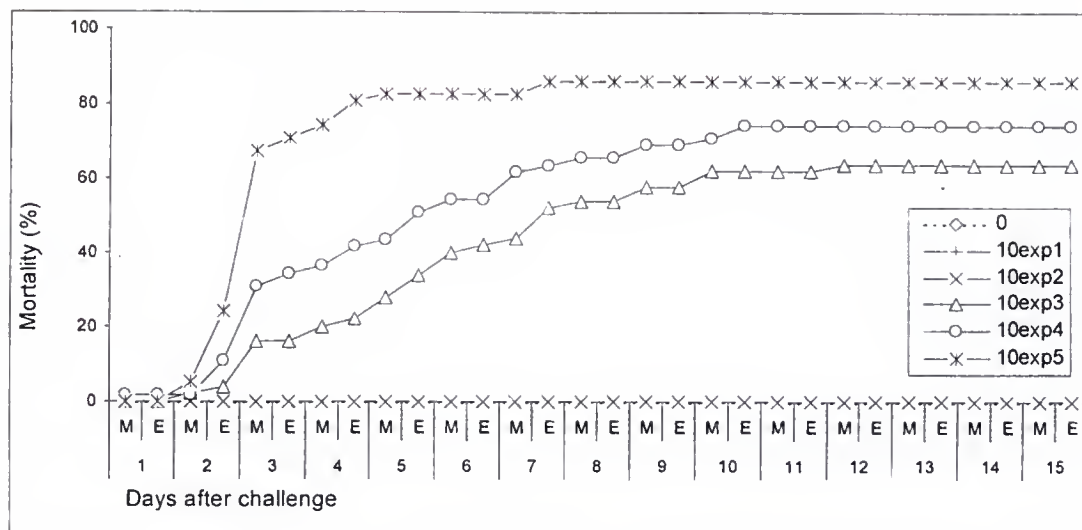


Figure 9-3. Average mortality rates (%) observed in experiment 3 with RTB shark. Number in abscissa refers to day after the challenge (day 0). M= morning (09:00), E= evening (21:00); 3 replicates were used per treatment and each tank was stocked with 25 fish.

Table 9-3. Average cumulative mortality (%) 14 days after bacterial challenge for the two experiments with rainbow shark, 3 replicates were used per treatment and each tank was stocked with 25 fish.

Specie	Trial n	Treatment bacteria/fish	Final Mortality %±SD	Significance
Rainbow shark (<i>E. erythrurus</i>)	4	Control	0±0	a
		1.5×10^3	45±12	b
		1.5×10^4	86±5	c
		1.5×10^5	98±3	d
		1.5×10^6	100±0	d
	5	Control	2±2	e
		1.5×10^1	2±3	e
		1.5×10^2	6±2	e
		1.5×10^3	21±2	f
		1.5×10^4	79±11	g
		1.5×10^5	100±0	h

In the treatment column the concentration of injected *S. iniae* (bacteria/mL) used for each trial are reported, and in the last column are reported the results of the Tukey's test. ($p_4 = 3.4 \times 10^{-12}$, $p_5 = 3.4 \times 10^{-12}$, $\alpha = 0.05$).

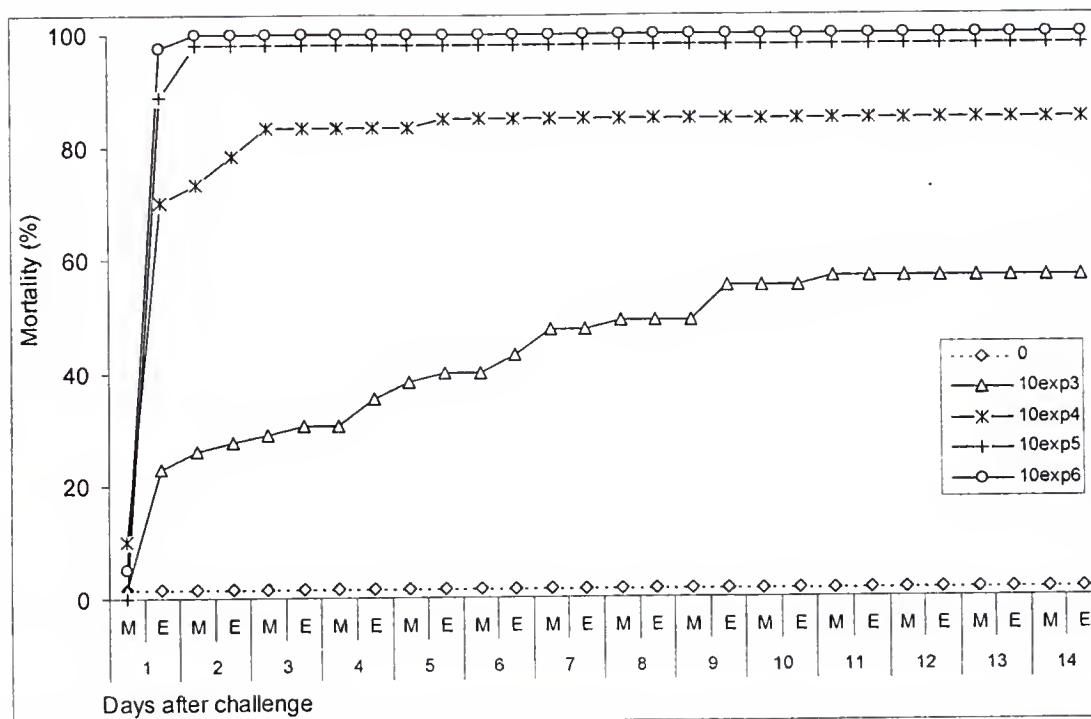


Figure 9-4. Average mortality rates (%) observed in the experiment 4 with rainbow shark. Number in abscissa refers to day after the challenge (day 0). M= morning (09:00), E= evening (21:00); 3 replicates were used per treatment and each tank was stocked with 25 fish.

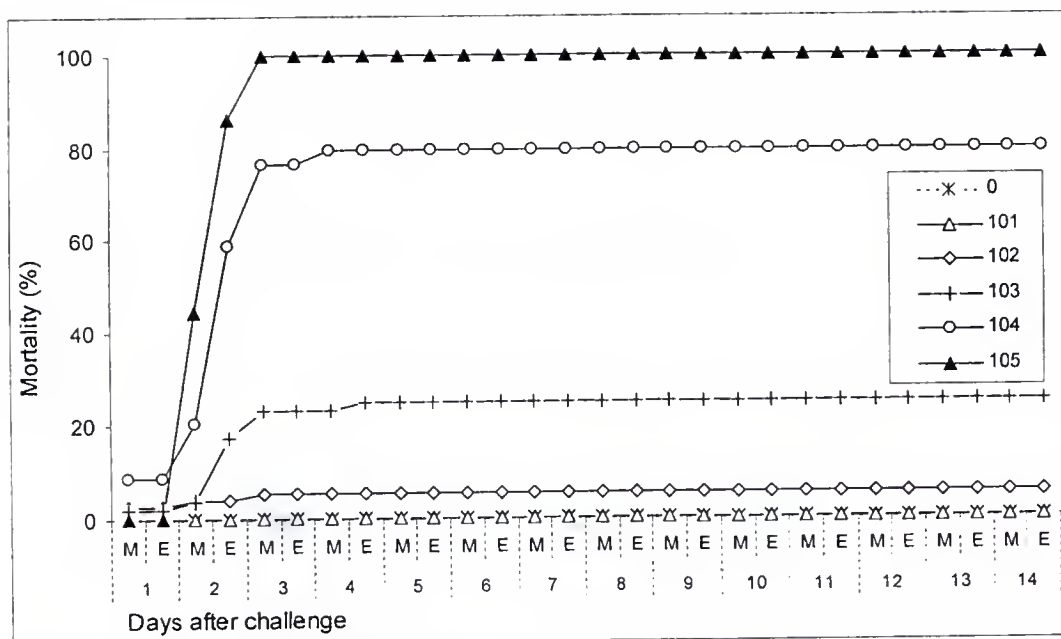


Figure 9-5. Average mortality rates (%) observed in the experiment 5 with rainbow shark. Number in abscissa refers to day after the challenge (day 0). M= morning (09:00), E= evening (21:00); 3 replicates were used per treatment and each tank was stocked with 25 fish.

CHAPTER 10
EFFICACY OF DIFFERENT VACCINE FORMULATIONS AGAINST *Streptococcus*
iniae AND ADMINISTRATION ROUTES IN RED-TAIL BLACK SHARK
(*Epalzeorhynchus bicolor*), FAM. CYPRINIDAE

10.1. Introduction

One of the main problems in aquaculture is maintaining fish in good health. High rearing density, poor water quality, poor feed quality, handling, and other stressors can inhibit the immune system making fish more susceptible to infections and causing economic losses due to mortality and drug applications. In the long run, prevention is more economical than treatment, and the risk of selecting for drug resistant strains of pathogens is reduced. Furthermore environmental and regulatory concerns may decrease the possibility of drug applications. Vaccination, good water quality, good management and good feed quality are some preventive strategies that should always be followed by fish farm facilities. Vaccines protect fish by inducing a specific immune response and an increased capacity to kill the pathogens by non-specific defense mechanisms. Vaccination confers protection against disease, not necessarily infection, and so persistent infection in an asymptomatic carrier state may be present. The process for vaccine development is very long and expensive, and for these reasons only vaccines targeting the major cultured fish species are currently on the market.

The purpose of this set of experiments was to develop and test the protection afforded by several formulations and applications of a vaccine against *Streptococcus iniae* in red-tail black shark (RTB shark), an economically important species for the Florida ornamental fish industry. This species is very susceptible to streptococcosis and

Florida ornamental fish industry. This species is very susceptible to streptococcosis and juveniles appear most vulnerable, especially after harvest from ponds. This susceptibility makes this species a good candidate for vaccine development. Because freshwater sharks are sold when they reach approximately 2.5-5 cm total length, farmers are interested in vaccinating juveniles primarily, and for these reasons we used this fish size in most of our research. Another goal of this research is to create a model for the development of vaccines for other ornamental fish species and for other bacterial diseases.

10.2. Materials and Methods

Experimental animals and systems. The research was conducted at the Tropical Aquaculture Laboratory, University of Florida (Ruskin, FL) using two recirculating systems with 25 tanks of 38-L volume. RTB shark juveniles (mean weight 1.1 ± 0.4 g and TL 5.0 ± 0.5 cm) were obtained from an ornamental fish farm in Hillsborough county (FL) where they were raised in ponds. A representative group of 30 individuals was measured at the beginning of the research. To verify the *S. iniae*-free status of the fish, brain and kidney tissues were sampled from 5 representative selected fish before each experiment. The samples were spread on Tryptic Soy Agar with 5% sheep's blood (Physician's Laboratory Supply, Troy, Michigan) and incubated at 37°C for 48 h.

Temperature, ammonia, and nitrite in the water systems were measured daily; alkalinity, hardness and pH once at week. The water in the system was maintained at $25.5 \pm 0.6^\circ\text{C}$. Ammonia and nitrite concentrations were always close to 0 mg/L. Alkalinity and hardness were 110 ± 10 mg/L and 115 ± 15 mg/L respectively; pH was 7.5.

Bacterial strain. Details of the bacterial isolation and batch broth culture were reported previously (Chapter 9).

Vaccine production. The Novartis-Aqua Health LTD. laboratory in Charlottetown, Canada using our *S. iniae* strain, manufactures a formalin-killed autogenous bacterin using their proprietary fermentation and downstream-processing methods. Three different formulations of the same vaccine were prepared: the basic one (W), and with addition of aluminum salt (A) or oil (O) as adjuvant. The recommended vaccine dose for injection was of 0.1 mL for fish bigger than 8 g. Because the injection of such volume is not possible in the 1 g fish due to small volume of the coelomic cavity, and based on pilot trials, we decided to vaccinate fish with a dose of 0.06 mL.

Experimental design. In this experiment the three vaccine formulations (W, A, O) were each tested by injection, and the basic formulation (W) was also tested by bath and oral administration.

Each tank was stocked with 25 fish. Eight tanks were randomly assigned for each treatment and for the control. Fish were vaccinated after a week of acclimation. In the three injection treatments, groups of 12 fish were anesthetized with 100 mg/L MS-222 and then injected with 0.06 mL of one of the vaccine formulations. In the bath treatment, fish were immersed for 1 minute in a vaccine dilution of 1:4 for a total volume of 1 L. In the oral treatment fish were fed frozen brine shrimp coated with vaccine for 3 weeks. To coat the brine shrimps with vaccine, an equal volume of vaccine was added to defrosted brine shrimp, and then the mixture was mixed and left to dry overnight at 30°C. Twice a day, fish were fed treated brine shrimp for a total daily dose of 3% body weight wet weight. Mortalities after vaccinations were recorded daily.

After 21 days, corresponding to 536 degree/days (degree/days is the result of the multiplication of the number of days between vaccination and bacterial challenge, and the average water temperature recorded in this period. Because the fish immune system is influenced by the temperature, the arbitrary measurement degree/days has been adopted to establish at a specific water temperature, which should be the minimal time period between vaccination and a possible exposure to a specific pathogen.), fish were challenged with 1.5×10^4 *S. iniae* cells/fish by an intracoelomic injection of 0.05 mL bacterial culture. Mortality was recorded for 12 days after challenge. During this period, dead fish were removed 3 times a day. Brain and kidney cultures of 70-80 % of the moribund or dead fish were taken for microbiological testing to attempt to verify the cause of mortality.

Before the challenge, at the end of the experiment, 12 representative fish from each treatment were preserved in 10% formalin. Also, a few dead fish collected in the first week after vaccination, were preserved in 10% formalin. Hematoxylin and eosin (H&E) or gram staining of tissue sections were prepared on a representative sample of 5 fish for each of the treatments. Photomicrographs of the histological sections were captured on computer using the capture system Moticap L312 (Motic Instruments, Richmond, B.C., Canada) and a video camera Ultrak (Ultrak, Lewisville, Texas) connected to the computer and to a light microscope with 100x or 400x magnification.

Statistical analysis. ANOVA analysis and Tukey's post hoc test were run for each experiment using the statistical program SPSS 12.0 (SPSS Inc., Chicago, IL). An asin(square root) transformation was performed on the mortality data expressed as

percentage. The results of the ANOVA analyses were considered significant at a $p\text{-value} < 0.01$; then the Tukey's test was considered at an $\alpha = 0.05$.

10.3. Results

Average mortalities between 17 and 21% were observed in the first 12 h after the injection of any of the three vaccine formulations (Figure 10-1 and Table 10-1). To determine if these mortalities were caused by side effects of the vaccine formulations or were “normal” mortality connected with the stress of injecting these small fish (1.3 g average weight), an ANOVA analysis was conducted comparing the mortalities observed in the first 12 h after the injection of each vaccine formulation with the mortalities observed in the first 12 h after the challenge by injection with *S. iniae*. As reported in fig. 1 and tab. 1, the mortality recorded after the vaccine injections was significantly different from the mortality observed after the bacterial injections. Only the injection of the oil formulation (OJv) appeared to cause no significant higher mortality than the injection of bacteria in the same treatment (OJb), even if the mortality was higher than the injection of bacteria in other treatments. The result of a t-test run between these two sets of data showed that in reality there is a significant difference ($p < 0.01$).

Similar results were observed for the mortalities observed in the period between the vaccination and the bacterial challenge (Table 10-1). This period of time was divided in two parts, the beginning of the second period coinciding with when mortalities were recorded in all treatments. In the first 10 days after vaccination, average mortalities between 29 and 31% were observed only in the treatment groups of fish vaccinated by injection (WJ, OJ, AJ). In the last 10 days lower average percent mortality, between 1 and 9%, were observed in all treatments, but high mortality (23%) was still observed in

fish vaccinated with the aluminum formulation. From the observation of aggressive behaviors among the fish, of bite marks on dead fish, of the recovery of half eaten bodies only in this period, and the common knowledge that sharks are aggressive fish; it is suspected that the mortalities observed in the second period were caused mainly by the aggressive behaviors among fish. These aggressive behaviors were not observed after the injection with *S. iniae*, most likely due to the related stress and subsequent induced infections.

Microscopic evaluation of H&E and gram-stained tissue sections of dead fish collected in the first week after intracoelomic vaccination revealed the presence of necrotic areas in the intestine, spleen and kidney. Large granuloma-like areas were observed only in the spleen, liver, anterior and posterior kidney of fish vaccinated with the aluminum formulation (Figure 10-2 and 10-3).

From the results of the vaccine test (Table 10-2 and Figure 10-4) it is possible to conclude that the mortality in the bath and oral route of vaccine administration was not significantly different from the control. Fish vaccinated with oil (OJ) and the aluminum (AJ) formulations had a significantly lower mortality ($15 \pm 13\%$ and $13 \pm 12\%$ respectively) compared to the control ($47 \pm 10\%$). The Tukey's test (tab. 9.2) showed that mortality of fish vaccinated with the basic formulation by injection (WJ) ($36 \pm 13\%$) was not significantly different from the control (C). However, the results of the t-tests run among this treatment and the control or the oil or aluminum injection treatments showed that the mortality in the fish vaccinated by injection with the basic formulation was significantly lower ($p < 0.01$) compared to the control group, but significantly higher ($p < 0.01$) compared to the oil or aluminum formulations.

10.4. Discussion

The results of the vaccine test demonstrated that both the oil and the aluminum formulations significantly protected the fish against *S. iniae* compared to the control. Even if the Tukey's test showed no significant difference in protection between the basic formulation and the control, the results of the t-tests run between this treatment (WJ) and the control (C) or the oil or aluminum injection treatments (OJ, AJ) showed that the basic formulation by injection significantly decreased fish mortality compared to the control group, but this level of protection was significantly lower than that observed with the oil or aluminum formulations. The bath and oral route of vaccine administration were not efficient and did not provide protection compared to the control.

The results of this series of experiments demonstrated that the vaccine produced with the same strain of *S. iniae* that we isolated, was effective in protecting the RTB shark. However, the results show that intracoelomic injection was the only administration method that provided protection with our protocol. The results of this research are in accordance with those in several papers which also demonstrated that the injection method was always either the only route or the route that provided greater and longer lasting protection against several bacterial diseases (Iida et al. 1982; Lin et al. 2000; Midtlyng et al. 1996a; 1996b). Several studies have demonstrated that intracoelomic injection of vaccine stimulates systemic production of antibody, whereas bath or oral vaccine administration may in some cases stimulate only mucosal immunity (Lin et al. 2000). The oral administration of microencapsulated antigens has been shown to be effective in some cases (Joosten et al. 1997). For example, in salmonids the protection given by enteric-coated antigen microspheres was as efficacious as injection of vaccine

(Piganelli et al. 1994). In rats, it has been demonstrated that oral administration of microcapsules significantly increased secretory IgA (O'Hagan et al. 1989).

Microcapsules can be absorbed by Peyer's patches and can be taken up by macrophages, and in this way stimulate systemic immunity (O'Hagan, 1992). Another advantage of using microcapsules is the possibility of including adjuvants.

The results of this research also demonstrated the effect and importance of adding an adjuvant to the basic vaccine formulation. In this research adjuvants dramatically increased the level of protection compared to the basic vaccine formulation. These results are in accordance with what has been reported by other researchers (Horne et al. 1984; Midtlyng, 1996). For example, the success of the vaccine against furunculosis has been attributed to the use of an adjuvant (Gudding et al. 1999).

Side effects, such as granuloma formation and other types of lesions, are a problem when using adjuvants, and this has been reported frequently (Horne et al. 1984; Midtlyng, 1996; Midtlyng et al. 1996ab). In this study we observed severe side effects due to the injection of the vaccine formulations. The aluminum formulation produced the worst effects causing higher cumulative mortality before the bacterial challenge compared to injection of the oil or base vaccine formulations, as well as the formation of extensive granuloma-like areas in several organs that were not observed in the other treatments (WJ, OJ). The oil formulation had a milder effect on the fish, also demonstrated by the observed faster recovery after the vaccine injection. Similar results were observed in trout (Horne et al. 1984) where a potassium alum vaccine caused high mortality, pathological changes related to chronic peritonitis, infiltration of macrophages in the ovary, pancreas and liver serosa with the following formation of epithelioid layers

around focal granuloma associated with the accumulation of the aluminum salt. These pathologies were more evident in 3 cm fish than in 20 cm fish. Growth depression was observed in adult fish. Interestingly, in salmonids it appears that oil formulations cause worse side effects than aluminum formulations (Midtlyng, 1996).

10.5. Conclusions

In this research we demonstrated the efficacy of a vaccine against *S. iniae* especially when adjuvants are added to the formulation. Unfortunately only the injection route of administration was efficient in providing protection for the fish. Clearly for juvenile fish the administration of vaccine by injection is not economically feasible, but the results of other experiments (Chapter 15) demonstrated that this route of administration might be used for broodstock. However, the results of this experiment are still important because this work is a first step toward the development of a challenge model and vaccine efficacy tests for other ornamental fish. As has been described previously, low efficacy may be observed in the first attempts to develop new vaccines, but the results of the first attempts are important in setting a baseline level of protection and for allowing improvement and development of future vaccines (Mitchell, 1999). The injection route of vaccine administration could be made feasible by the development of an automatic vaccination machine that could be used for other ornamental fish.

The objectives of additional research, to be described in the next chapters, will be to reduce or eliminate the substantial mortalities recorded after the injection of the vaccine, especially for the aluminum formulation. Due to the small size of the fish, antibody levels could not be measured. Our future research with broodstock will try to accomplish this task.

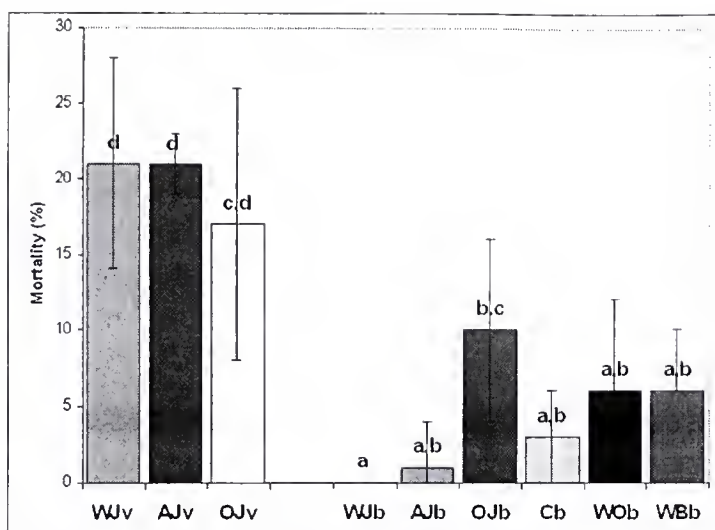


Figure 10-1. Average mortality (%) recorded in the first 12 hours after injection of the 3 vaccine formulations or of the bacterial challenge (1.5×10^4 bacteria/fish). Bars represent the standard deviation. The letters above the bars represent the level of the significant difference among treatments. $p_1 = 7.3 \times 10^{-10}$; $\alpha = 0.05$. Abbreviations: C= control, WO= basic formulation by oral administration, WB= basic formulation by bath, WJ= basic formulation by injection, AJ= aluminum formulation by injection, OJ= oil formulation by injection, v= vaccine injection, b= bacterial injection; 8 replicates were used per treatment and each tank was stocked with 25 fish.

Table 10-1. The average mortality (%) recorded in the first 12 hours after injection of the vaccine formulations or of the bacterial challenge (1.5×10^4 bacteria/fish) and the average mortality (%) in the first and second 10 days between vaccination and bacterial challenge. The letters in the columns correspond to the results of the two Tukey's tests run between the second-third and fourth-fifth columns.

Treatment	Average mortality % $\pm$ SD			
	First 12 hours after injection		Days after vaccination and before bacterial challenge	
	Vaccine	<i>S. iniae</i>	1-10	11-20
C		3 $\pm$ 4 a,b	0 $\pm$ 0 e	9 $\pm$ 3 f,g
WO		6 $\pm$ 6 a,b	0 $\pm$ 0 e	1 $\pm$ 2 e,f
WB		6 $\pm$ 4 a,b	0 $\pm$ 0 e	8 $\pm$ 6 f,g
WJ	21 $\pm$ 7 d	0 $\pm$ 0 a	30 $\pm$ 16 h	6 $\pm$ 3 e,f,g
OJ	17 $\pm$ 9 c,d	10 $\pm$ 6 b,c	29 $\pm$ 19 h	9 $\pm$ 10 e,f,g
AJ	21 $\pm$ 2 d	1 $\pm$ 3 a,b	31 $\pm$ 6 h	23 $\pm$ 21 g,h

($p_1 = 7.3 \times 10^{-10}$ and $p_2 = 3.9 \times 10^{-07}$, $\alpha = 0.05$); 8 replicates were used per treatment and each tank was stocked with 25 fish. Abbreviations: C= control, WO= basic formulation by oral administration, WB= basic formulation by bath, WJ= basic formulation by injection, OJ= oil formulation by injection, AJ= aluminum formulation by injection.

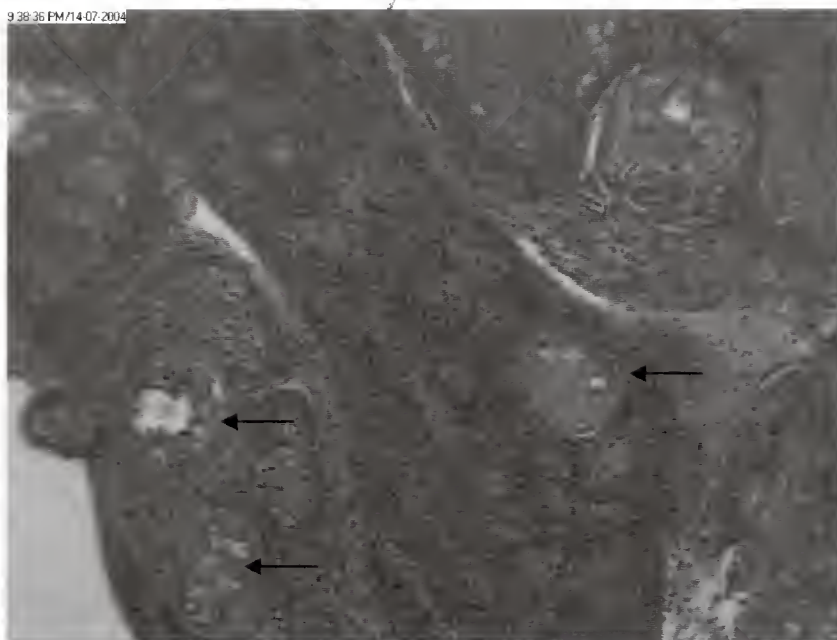


Figure 10-2. H&E staining of a posterior kidney section of a fish injected with the aluminum formulation (100x magnification). Note arrows showing granuloma-like lesions.

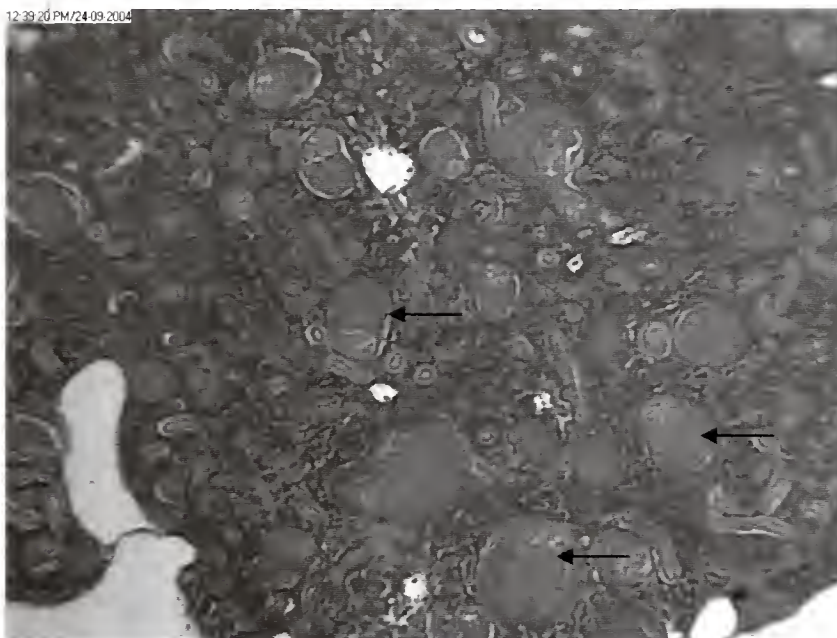


Figure 10-3. H&E staining of a spleen section of a fish injected with the aluminum formulation (100x magnification). Note arrows showing granuloma-like lesions.

Table 10-2. Total average mortality (%) recorded for 12 days after challenge with *S. iniae* (1.5×10^4 bacteria/fish). In the third column the level of the significant difference among treatments as resulted from the Tukey's test are reported.

Treatment	Average Mortality %	Significance
C	47 ± 10	a
WO	50 ± 12	a
WB	49 ± 15	a
WJ	36 ± 13	a
OJ	15 ± 13	b
AJ	13 ± 12	b

($p = 2.0 \times 10^{-06}$, $\alpha = 0.05$). Abbreviations: C= control, WO= basic formulation by oral administration, WB= basic formulation by bath, WJ= basic formulation by injection, OJ= oil formulation by injection, AJ= aluminum formulation by injection; 8 replicates were used per treatment.

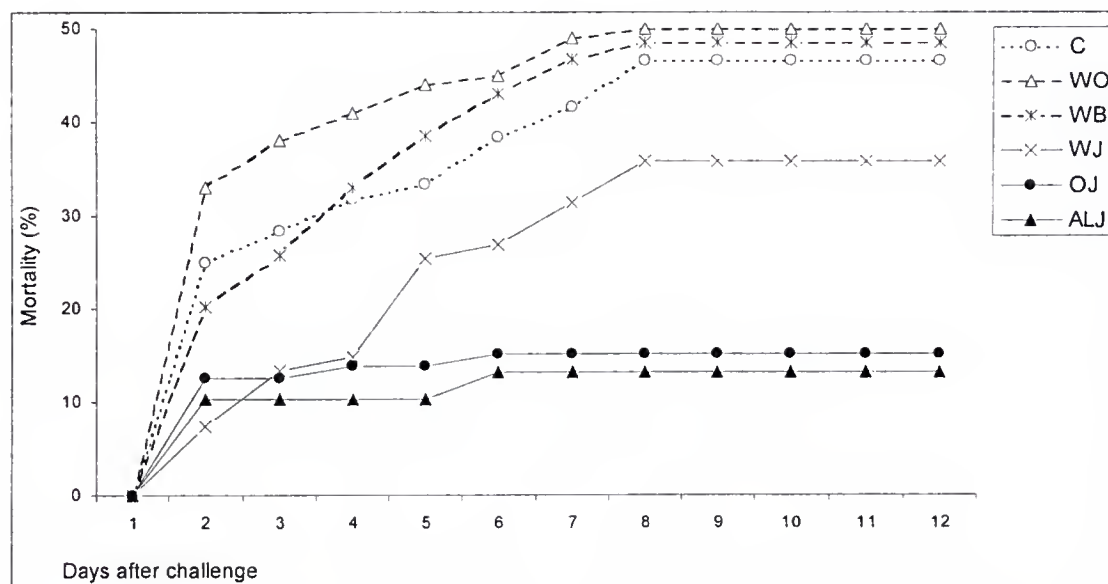


Figure 10-4. Average mortality rate (%) recorded for 12 days after challenge with *S. iniae* (1.5×10^4 bacteria/fish). Abbreviations: C= control, WO= basic formulation by oral administration, WB= basic formulation by bath, WJ= basic formulation by injection, AJ= aluminum formulation by injection, OJ= oil formulation by injection; 8 replicates were used per treatment.

CHAPTER 11
IMPROVEMENT OF VACCINATION STRATEGIES AGAINST *Streptococcus iniae*
IN JUVENILE RED-TAIL BLACK SHARK (*Epalzeorhynchus bicolor*), FAM.
CYPRINIDAE

11.1. Introduction

Among the major goals of our vaccine research are to develop a challenge and vaccine efficacy test models for the commercial ornamental fish industry in Florida. We developed a challenge model against *Streptococcus iniae* for red-tail black shark (*Epalzeorhynchus bicolor*, fam. Cyprinidae) and rainbow shark (*E. bicolor*) and we tested the efficacy of three formulations of a formalin killed bacterial vaccine (Chapters 9, 10). The vaccines were produced by Novartis-Aqua Health LTD., Charlottetown, Canada, using the strain of *S. iniae* isolated from a clinical case (as described previously).

In previous experiments we observed that only the vaccine formulations with aluminum or oil as an adjuvant protected red-tail black shark (RTB shark) against *S. iniae* with a relative percent survival (RPS) of 72 and 68 %, respectively (Chapter 10). However, significant mortalities, presumably due to toxic side effects, were recorded after the injections of both vaccine formulations in the fish. It was hypothesized that the observed mortalities were due to the small size of fish used in the experiments (WT 1.3 g, TL 5.2 cm) relatively to the volume of injected vaccine (0.06 mL/fish). Other experiments were designed to verify if a lower injected vaccine dose would still provide a good level of protection and at the same time decrease or eliminate the mortalities presumably due to vaccine side effects. In a second series of experiments we tested if the

administration of the aluminum formulation by bath would be an effective vaccination route.

11.2. Materials and Methods

Experimental animals and systems. The research was conducted at the Tropical Aquaculture Laboratory, University of Florida (Ruskin, FL) using two recirculating systems with 25 tanks of 38-L volume. RTB shark juveniles (mean weight 1.2 ± 0.3 g and TL 5.0 ± 0.3 cm) were obtained from an ornamental fish farm in Hillsborough county (FL) where they were raised in ponds. A representative group of 30 individuals for each species was measured at the beginning of the research. To verify the *S. iniae*-free status of the fish, brain and kidney tissues were sampled from 5 representative selected fish before each experiment. The samples were spread on Tryptic Soy Agar with 5% sheep's blood (Physician's Laboratory Supply, Troy, Michigan) and incubated at 37°C for 48 h.

Temperature, ammonia, and nitrite in the water systems were measured daily; alkalinity, hardness and pH once at week. The water in the system was maintained at $25.5 \pm 0.6^\circ\text{C}$. Water ammonia and nitrite concentrations were always close to 0 mg/L. Alkalinity and hardness were 110 ± 10 mg/L and 115 ± 15 mg/L respectively; pH was 7.5.

Bacterial strain. Details of the bacterial isolation and batch broth culture were reported previously (Chapter 9).

Vaccine production. Details of the vaccines production were reported previously (Chapter 10). In these experiments only the aluminum and oil formulations were used.

First experimental design. This experiment was designed to verify if a lower injected vaccine dose would still provide a good level of protection and at the same time

decrease or eliminate the mortalities presumably due to vaccine side effects. Five treatments were chosen in which fish were vaccinated by intracoelomic injection of 0.01 (A1), 0.03 (A3), 0.06 mL (A6) of the aluminum formulation or with 0.01 (O1), 0.03 mL (O3) of the oil formulation. A dose of 0.06 mL of the oil formulation was not tested due to the limited number of tanks in the system (25), the cost of the fish and the results of the preceding experiment (Chapter 10) where the level of protection and mortality due to toxic effects of the vaccines were the same injecting 0.06 mL of the oil or aluminum formulations. However, 25 fish kept in one tank during the experiment, were vaccinated with 0.06 mL of the oil formulation (O6) for histological analysis. The control group was injected with 0.05 mL of sterile BHI (same volume used for the bacterial challenge).

Each tank was stocked with 25 fish. Four tanks were randomly assigned for each of the 5 treatments and for the control. Fish were vaccinated after a week of acclimation. Groups of 12 fish were anesthetized with 100 mg/L MS-222 and then injected with vaccine. Mortalities after vaccination were recorded daily.

After 21 days, corresponding to 536 degree/days, fish were challenged with 1.5×10^5 *S. iniae* cells/fish by an intracoelomic injection of 0.05 mL bacterial culture. Fish were challenged with a higher bacteria dose than in the first vaccine experiment (Chapter 10) for trying to observe a possible difference in vaccine efficacy between the aluminum and oil formulations. Mortality was recorded for 12 days after challenge. During this period, dead fish were removed 3 times per day. Brain and kidney samples of 70-80% moribund or dead fish were taken for microbiological testing to attempt to verify the cause of mortality. Before the challenge and at the end of the experiment, 12 representative fish from each treatment were preserved in 10% formalin. Hematoxylin

and eosin (H&E) or gram staining of tissue sections were prepared on a representative sample of 8 fish from the A6 treatment group, the control group, and O6 group.

Photomicrographs of the histological sections were captured on computer using the capture system Moticap L312 (Motic Instruments, Richmond, B.C., Canada) and a video camera Ultrak (Ultrak, Lewisville, Texas) connected to the computer and to a light microscope with 100x or 400x magnification.

Second experimental design. Based on the results of first experiment, a second trial was run to test if the administration of the aluminum formulation by bath would be an effective vaccination route. Fish were vaccinated with the aluminum formulation by intracoelomic injection (0.04 mL/fish) or by bath (1 minute in a vaccine dilution of 1:4). Control fish were kept untouched. The experimental protocol used in the first experiment was also used in this study.

Statistical analysis. ANOVA analysis and Tukey's post hoc test were run for each experiment using the statistical program SPSS 12.0 (SPSS Inc., Chicago, IL). An asin(square root) transformation was performed on the mortality data expressed as percentage. The results of the ANOVA analyses were considered significant at a $p\text{-value} < 0.01$; then the Tukey's test was considered at an $\alpha = 0.05$. Relative percent survival (RPS) was calculated with the formula:

$$\text{RPS} = \left[1 - \frac{\text{treatment mortality (\%)}}{\text{control mortality (\%)}} \right] * 100$$

11.3. Results

The reduced dosage concentration of vaccine formulations (0.01 and 0.03 mL of the aluminum and oil formulations) resulted in no significantly different mortalities in the

first 12 h after injection (Table 11-1) compared to the control group (average mortalities between 3 and 9%). In contrast (see Chapter 10), use of 0.06 mL of the aluminum formulation caused significantly greater mortality ($26 \pm 9\%$).

Similar results were observed for the mortality in the period between vaccination and bacterial challenge (Table 11-1). In this period significantly higher mortality than the control group ($0 \pm 0\%$) was observed only in fish vaccinated with 0.06 mL of the aluminum formulation ($19 \pm 4\%$).

The mortalities observed for 12 days after the challenge with *S. iniae* (Table 11-2, Figure 11-1) showed that when vaccine doses less than 0.06 mL are injected, i.e. at dosage rates of 0.01 and 0.03 mL/fish, the fish mortality increased significantly. Furthermore, for the same volume of injected vaccine, the mortality observed with the aluminum formulation was significantly lower than the observed in the oil formulation. The ANOVA analyses run for both formulations (aluminum and oil) between the 0.01 and 0.03 mL doses failed to show any significant difference in mortality due to the low power of the analyses (0.3 for the oil and 0.14 for the aluminum). However, in this experiment we were not interested in drawing a vaccine dose/protection level curve, but to test if it was possible to eliminate or decrease the toxic side effects while maintaining a sufficient level of protection with a dosing volume of less than 0.06 mL vaccine in a 1 g fish. In order to construct a vaccine dose/protection level curve, more replicates and more vaccine doses would be required.

Microscopic analysis of histological samples of fish not challenged with *S. iniae* revealed that the oil or aluminum formulations affected the major lymphoid organs (kidney and spleen) of sharks differently. In H&E stained sections of all unvaccinated

fish analyzed, a few small macrophage aggregates can be seen in the spleen (Figure 11-2), but not in the posterior kidney (Figure 11-3). In H&E stained sections of all fish vaccinated with the oil formulation analyzed, larger macrophage aggregates can be seen in the spleen (Figure 11-4), but the posterior kidney is similar to the control fish (Figure 11-5). When H&E staining of sections of all fish vaccinated with the aluminum formulation analyzed, several extensive granuloma-like lesions could be seen in the spleen (Figure 11-6) and posterior kidney (Figure 11-7). Such lesions are more evident in gram stained sections of the spleen (Figure 11-8). Similar formations were also observed in the liver (Figure 11-9). In gram stained sections of fish that survived to the *S. iniae* challenge, several basophilic spot corresponding to nuclei of lymphoid cells are visible inside the granuloma-like formation (Figure 11-10).

In the second experiment (Figure 11-11) the mortality of fish vaccinated by bath with the aluminum vaccine was not significantly different from the control group. In contrast, the mortality of fish injected with of 0.04 mL of the aluminum formulation was significantly lower than that of the control group. In the period between vaccination and bacterial challenge no mortality was observed in fish vaccinated by bath or injection.

11.4. Discussion

The results of the first experiment demonstrated that it is possible to eliminate the presumptive toxic side effects of the vaccine observed in previous experiments (Chapter 10) while still maintaining a certain level of protection. Only a detailed economic analysis can prove if this level of protection justifies the expense of vaccine application. However, the results of this study do provide a beginning template on which to build our model of vaccine efficiency trials for ornamental fish. Side effects, including lesions and

granulomas, appear to be a problem when using adjuvant, as has been reported repeatedly (Horne et al. 1984; Midtlyng, 1996; Midtlyng et al. 1996ab). Severe side effects similar to the ones we reported were observed in 3 cm trout (Horne et al. 1984) vaccinated with a potassium alum formulation. These fish showed high mortality, pathological changes connected to chronic peritonitis, infiltration of macrophages in the ovary, pancreas and liver serosa with the subsequent formation of epithelioid layers around focal granuloma associated with the accumulation of the aluminum salt.

From the results of this experiment, it appears that the aluminum formulation confers a higher level of protection level than the oil formulation. This hypothesis seems to be confirmed by the results of our other experiments with broodstock RTB and rainbow sharks (Chapters 12 and 15). The oil and the aluminum formulation had different effects on the lymphoid organs (spleen and kidney) of the fish: the oil formulation appeared to stimulate the formation of larger macrophage aggregates in the spleen, whereas the aluminum formulation also stimulated the formation of several large granuloma-like lesions not only in the spleen and posterior kidney, but also in the liver. These lesions appear to be involved in the immune response of the fish at some point, as demonstrated by the observation of gram positive streptococci inside these granulomas (Figure 11-11) after bacterial challenge. It is possible that these differing impacts on the immune system by the two formulations may be correlated with the protection level given by the same formulations. Interestingly, in salmonids, oil adjuvant vaccines are more effective than aluminum formulations (Midtlyng et al., 1996ab; Midtlyng, 1996; Nordmo and Ramstad, 1997). However the particle size of vaccines must also be considered for an accurate comparison of the efficacy among different formulations. In

fact, particles of different sizes are taken up by macrophages differently and this affects the degree of stimulation of the immune system (Muller and Harnisch, 1998).

In the second experiment the administration of the aluminum vaccine by bath did not protect the fish from the bacterial challenge, a fact that was observed in a preceding experiment (Chapter 10). It is possible that due to the size of the *Streptococcus* or for other reasons related with its nature or other properties, fish do not manage to assimilate enough bacteria to stimulate the immune system during the vaccination procedure.

11.5. Conclusions

By decreasing dosage concentration, we were able to improve the vaccination protocol by injection for small ornamental fish. Even if this protocol is not economically feasible for a commercial operation without an automatic injection machine, it does represent a step forward in our attempt to develop a challenge model and vaccine efficacy tests for new ornamental fish vaccines. As has been described in previous chapters, low efficacy is often observed in the first attempt to develop new vaccines, but the results of the first attempts are important for setting a baseline level of protection and for allowing improvement and development of future vaccines (Mitchell, 1999). However, the vaccine formulations can be used for vaccinating broodstock of ornamental fish, as demonstrated in other experiments (Chapter 15).

The results of this research show that in the RTB shark aluminum adjuvant stimulates a higher level of protection than oil adjuvant, and different effects on the immune organs can be seen after the vaccination with either formulation.

Table 11-1. Average mortality (%) recorded in the first 12 hours after injection of different volumes of the vaccine formulations (BHI for the control group), and in the period between vaccination and bacterial challenge. The letters in the columns correspond to the significant levels based on two Tukey's tests.

Treatment	Average mortality % $\pm$ SD	
	First 12 hours after injection	Between vaccination and challenge
C	9 $\pm$ 6 a	0 $\pm$ 0 c
O1	3 $\pm$ 5 a	0 $\pm$ 0 c
O3	6 $\pm$ 6 a	5 $\pm$ 5 c
A1	9 $\pm$ 5 a	0 $\pm$ 0 c
A3	3 $\pm$ 3 a	1 $\pm$ 2 c
A6	26 $\pm$ 9 b	19 $\pm$ 4 d

($p_1 = 4.6 \times 10^{-4}$, $p_2 = 3.7 \times 10^{-7}$, $\alpha = 0.05$). Abbreviations: C= control, A= aluminum formulation, O= oil formulation; numbers correspond to the volume of vaccine (0.01, 0.03, 0.06 mL) injected in each treatment; 4 replicates were used per treatment and each tank was stocked with 25 fish.

Table 11-2. Total average mortality (%) in fish injected with different vaccines doses recorded for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish). In the third column the levels of the significant difference among treatments as resulted from the Tukey's test are reported.

Treatment	Average Mortality	RPS	Significance
	% $\pm$ SD	% $\pm$ SD	
C	98 $\pm$ 3	0	a
O1	84 $\pm$ 7	14	b
O3	75 $\pm$ 7	23	b
A1	60 $\pm$ 11	39	c
A3	54 $\pm$ 3	45	c
A6	30 $\pm$ 7	69	d

($p = 6.2 \times 10^{-10}$, $\alpha = 0.05$); 4 replicates were used for treatment and each tank was stocked with 25 fishes. Abbreviations: RPS= relative percent survival, C= control, A= aluminum formulation, O= oil formulation; numbers correspond to the volume of vaccine injected in each treatment (0.01, 0.03, 0.06 mL); 4 replicates were used per treatment and each tank was stocked with 25 fish.

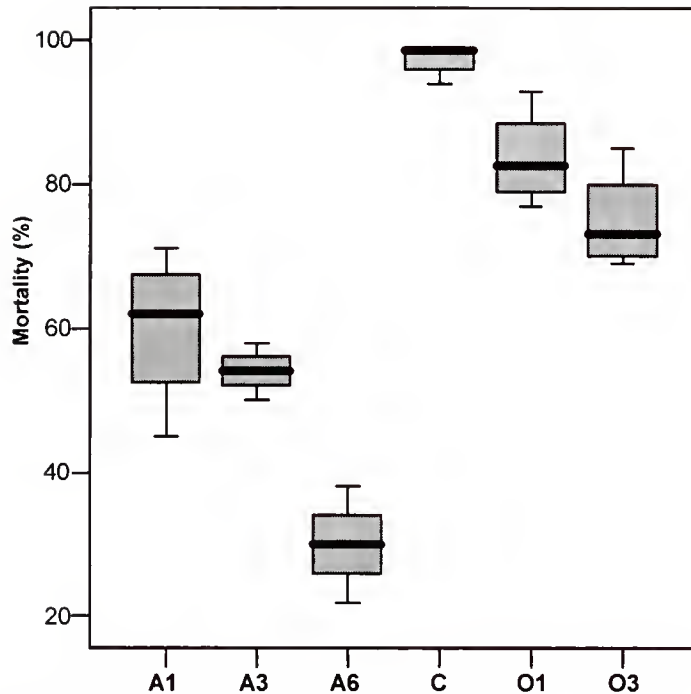


Figure 11-1. Box plot of the mortality rate (%) in fish injected with different vaccines doses recorded for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish). Boxes and bars respectively represent the distribution of 50% and 100% of data. Abbreviations: C= control, A= aluminum formulation, O= oil formulation; numbers correspond to the volume of vaccine injected in each treatment (0.01, 0.03, 0.06 ml).

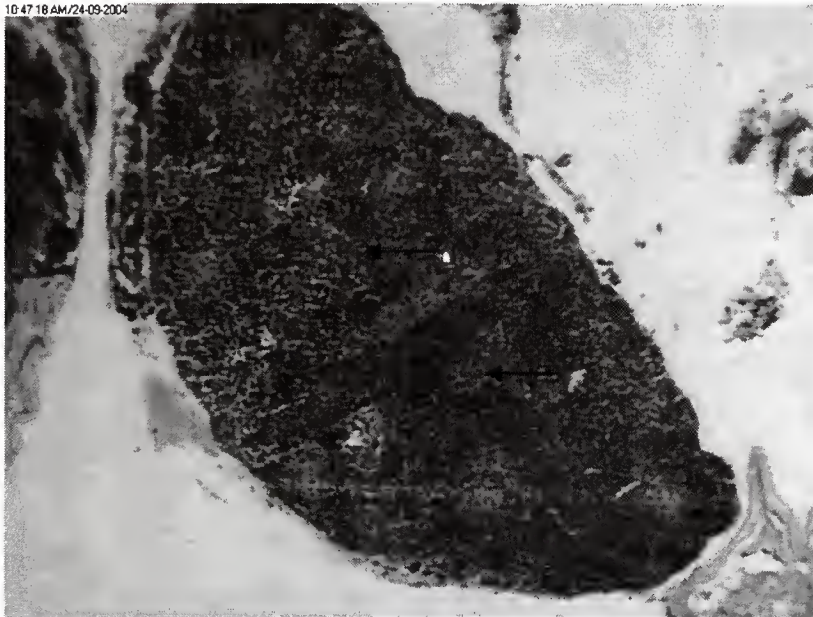


Figure 11-2. H&E staining of a spleen section of a non-vaccinated fish (100x magnification). Note arrows showing macrophage aggregates.

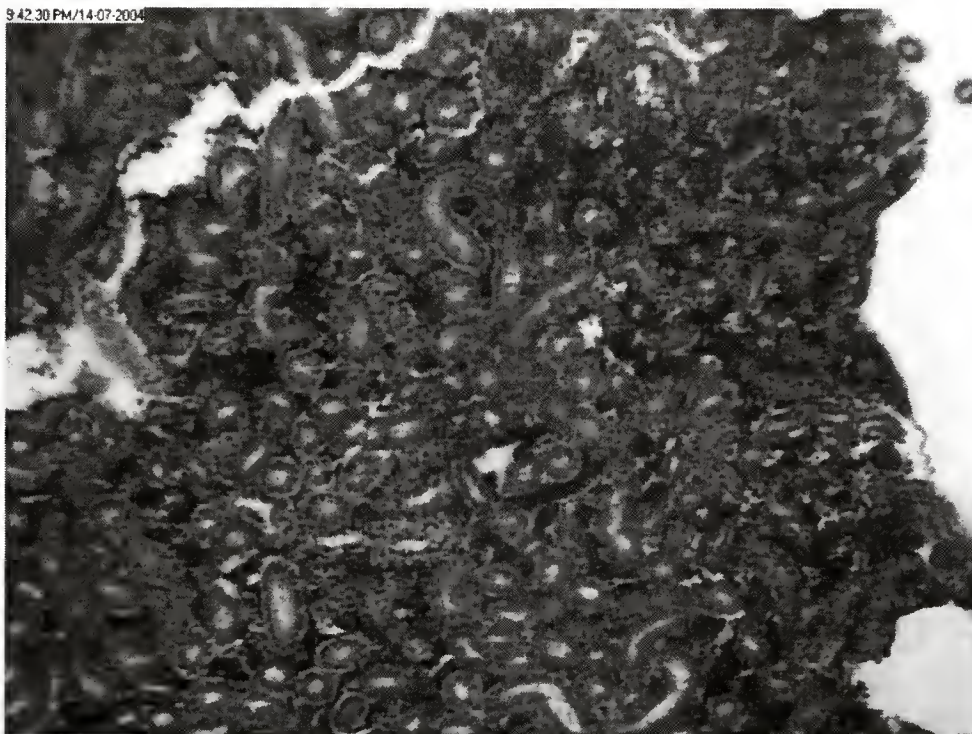


Figure 11-3. H&E staining of a posterior kidney section of a non-vaccinated fish (100x magnification).

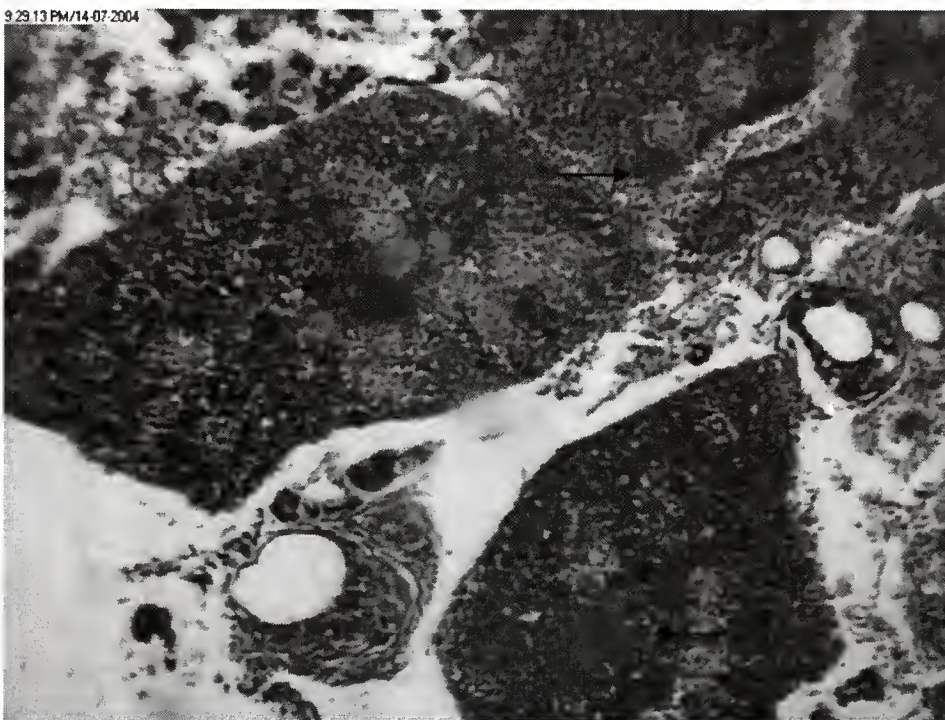


Figure 11-4. H&E staining of a spleen section of a fish injected with the oil formulation (100x magnification). Note arrows showing macrophage aggregates.



Figure 11-5. H&E staining of a posterior kidney section of a fish injected with the oil formulation (100x magnification).



Figure 11-6. H&E staining of a spleen section of a fish injected with the aluminum formulation (100x magnification). Note arrows showing granuloma-like lesions.

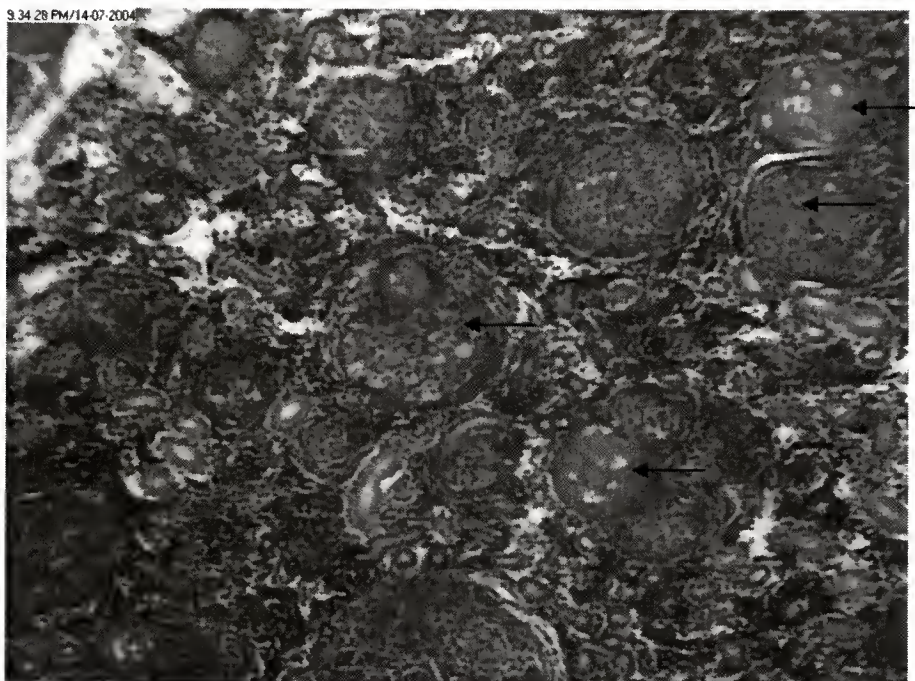


Figure 11-7. H&E staining of a posterior kidney section of a fish injected with the aluminum formulation (100x magnification). Note arrows showing granuloma-like lesions.

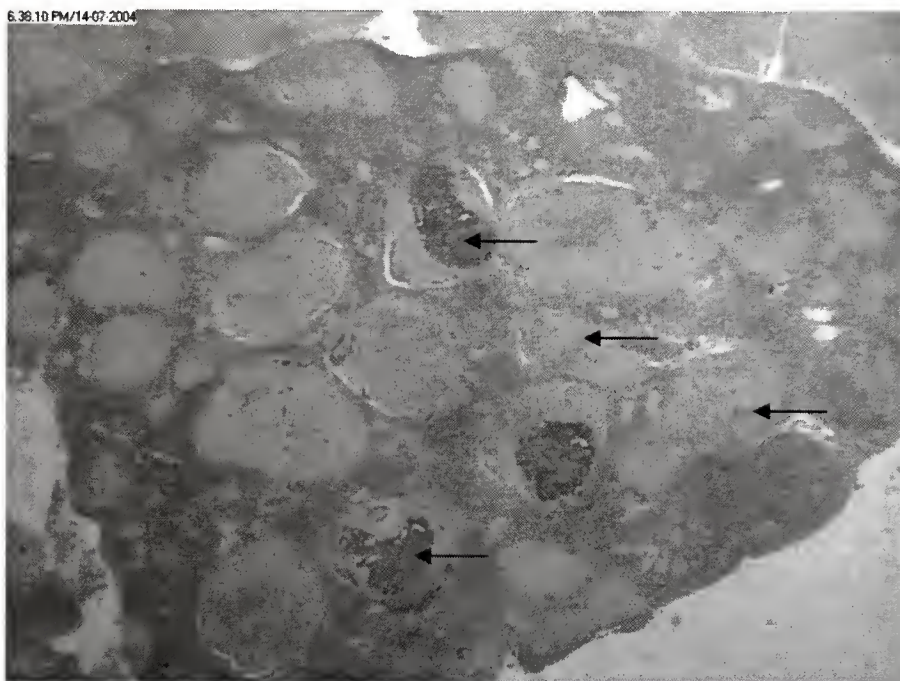


Figure 11-8. Gram staining of a spleen section of a fish injected with the aluminum formulation (100x magnification). Note arrows showing granuloma-like lesions.

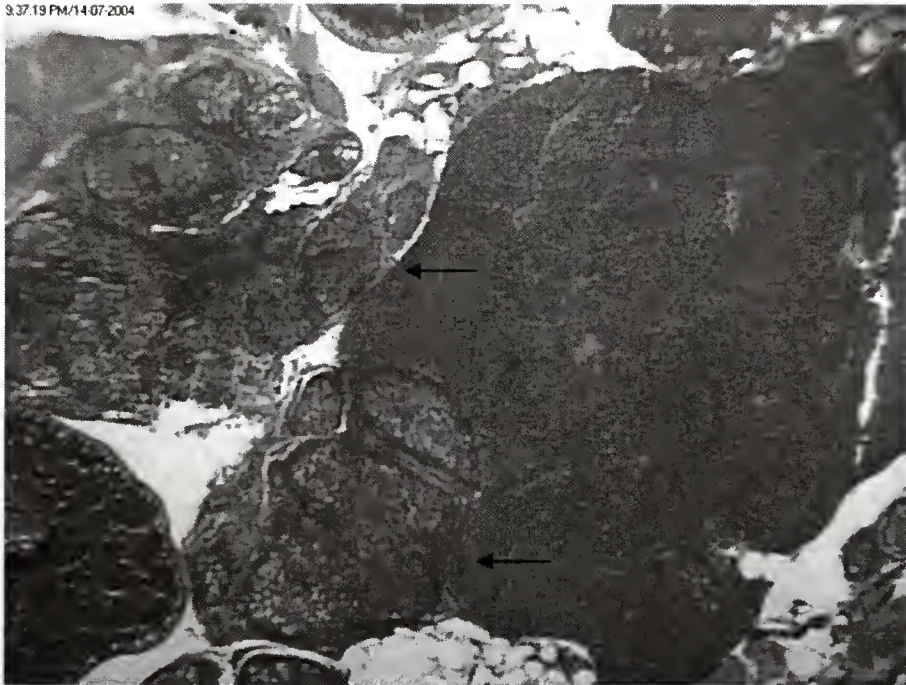


Figure 11-9. H&E staining of a liver section of a fish injected with the aluminum formulation (100x magnification). Note arrows showing granuloma-like lesions.

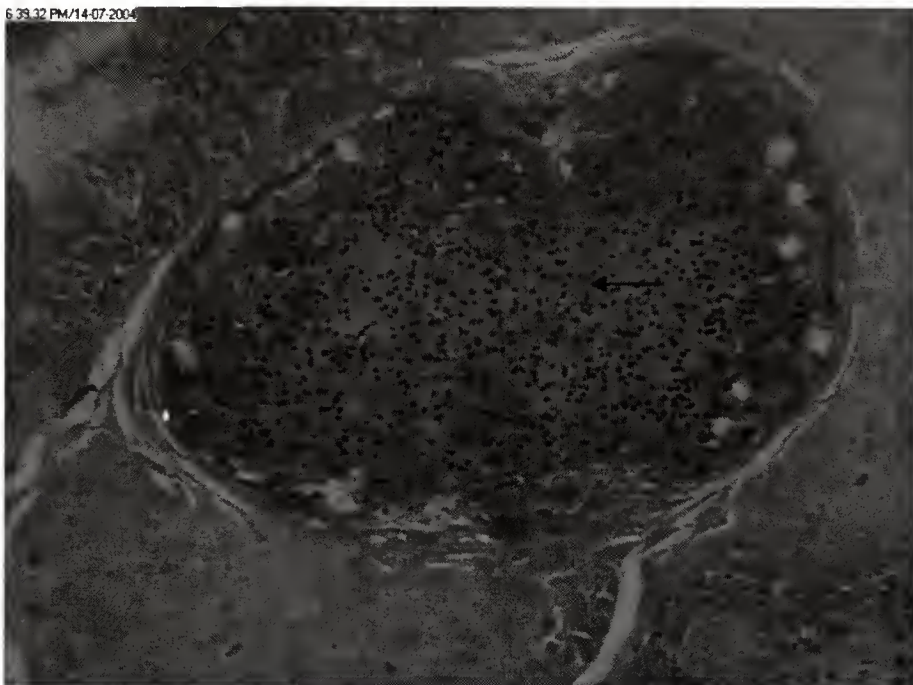


Figure 11-10. Gram staining of a granuloma-like formation in the spleen section of fish injected with the aluminum formulation (400x magnification). The section was prepared with a fish that survived the *S. iniae* challenge. Several basophilic spot corresponding to nuclei of lymphoid cells are visible inside the granuloma-like formation (indicated by the arrow).

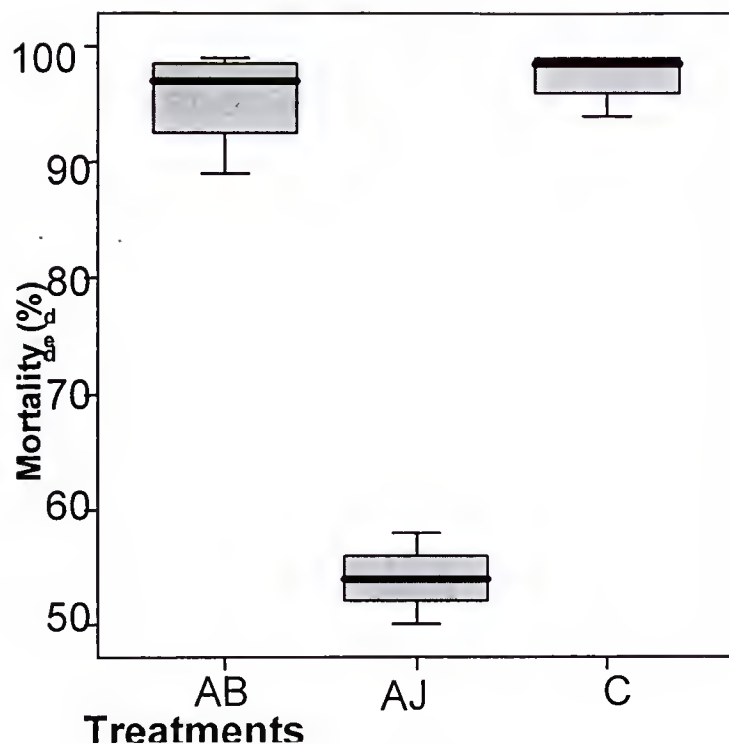


Figure 11-11. Box plot of the mortality rate (%) recorded in fish vaccinated with the aluminum formulation by bath or injection for 12 days after challenge with *S. iniae* (1.5×10^4 bacteria/fish). Boxes and bars respectively represent the distribution of 50% and 100% of data. ($p = 1.9 \times 10^{-6}$, $\alpha = 0.05$); 4 replicates were used per treatment and each tank was stocked with 25 fish. Abbreviations: C= control, AB= aluminum formulation by bath, AJ= aluminum formulation by injection.

CHAPTER 12
DIFFERENCES IN LEVELS OF PROTECTION PROVIDED BY DIFFERENT
VACCINE FORMULATIONS AGAINST *Streptococcus iniae* AMONG JUVENILE
AND ADULT FISH OF RAINBOW SHARK (*Epalzeorhynchus erythrinus*) AND
RED-TAIL BLACK SHARK (*E. bicolor*), FAM. CYPRINIDAE

12.1. Introduction

Differences in disease resistance due to genetic variations among closely related species of fish or among populations of the same species have been reported (Chevassus and Dorson, 1990; Chin et al. 2004; Dorson et al. 1991; Fjalestad et al. 1993; Gjedrem et al. 1991; Gjedrem and Gjoen, 1995; Wieger et al. 1996). It is known from studies in mammals, that a limited number of major genes, including the major histocompatibility complex (MHC), are involved in the regulation of the immune response and are responsible for differences in disease resistance among individuals of the same species. Recently, genes related to MHC class I and class II have been sequenced and described in salmonids (Knight et al. 1998; Shum et al. 2001), in carp, in zebrafish, and in a number of other cyprinid and non-cyprinid species (Stet et al. 1998). The observed absence of linkage between MHC class I and class II in fish would allow for an independent segregation of immunological traits associated with cytotoxic responses to virus infection (class I) or humoral responses to bacteria (class II) (Van Muiswinkel et al. 1999). Also other genes involved in disease resistance have been detected in fish, such as the natural resistance-associated macrophage protein (NRAMP) identified and characterized in carp (Saeij et al. 1999).

Sharks are economically important species for Florida's ornamental fish industry,

and are very susceptible to streptococcosis. In previous studies we developed a challenge model for *Streptococcus iniae* infection in red-tail black shark (RTB shark) and rainbow shark (Chapter 9); we tested the efficacy of three formulations of a formalin killed bacterial vaccine administered either by injection, bath or orally in RTB shark (Chapters 10 and 11). In this study we wanted to investigate whether or not a difference existed in the vaccine efficacy between these two closely related species of sharks, and between juveniles and adult fish.

12.2. Materials and Methods

Experimental animals and systems. The research was conducted in the Tropical Aquaculture Laboratory, University of Florida (Ruskin, FL), and in an ornamental fish farm in Hillsborough county (FL). RTB and rainbow shark juveniles (respectively 1.1 ± 0.4 g WT, 5.1 ± 0.4 cm TL and 1.1 ± 0.1 g WT, 5.0 ± 0.2 cm TL) and adult (respectively 21.2 ± 5.7 g WT, 12 ± 0.8 cm TL and 20.4 ± 4.2 g WT, 10.2 ± 0.5 cm TL) were obtained from an ornamental fish farm in Hillsborough county (FL). In the farm, fish were kept in a flow through system in 3,000 L vats or in ponds; in the laboratory fish were kept in recirculating systems with 25 tanks of 38-L volume. A representative group of 30 individuals for species was measured at the beginning of the research.

In all experiments in the laboratory water temperature, ammonia, and nitrite were measured daily; alkalinity, hardness and pH once at week. On the farm all water parameters were measured weekly. The water in the laboratory was maintained at $25.5 \pm 0.6^{\circ}\text{C}$ and in the vats at the farm at $26 \pm 1^{\circ}\text{C}$. Water ammonia and nitrite concentrations were always close to 0 (mg/L); in the laboratory alkalinity and hardness

were 110 ± 10 mg/L and 115 ± 15 mg/L respectively; pH was 7.5; similar values were measured at the farm.

Bacterial strain. Details of the bacterial isolation and batch broth culture were reported previously (Chapter 9).

Vaccine production. Details of the vaccines production were reported previously (Chapter 10).

First experimental design. This experiment was designed to investigate whether or not a difference existed in the vaccine efficacy between RTB and rainbow shark juveniles. Rainbow shark were vaccinated with the basic (W), aluminum (A) or oil (O) formulations by injection; RTB shark were vaccinated only with the aluminum (A) formulation due to the cost of the fish and the results of the preceding experiment (Chapters 10 and 11). The control groups for each species were injected with 0.04 mL of sterile BHI. Rainbow shark were also vaccinated by bath (1 minute in a vaccine dilution of 1:4) with the basic (W) or aluminum (A) formulations.

Juvenile sharks of both species were stocked at a density of 25 fish/tank and four tanks were randomly assigned for each treatment and for the controls. Fish were vaccinated after a week of acclimation. Groups of 12 fish were anesthetized with 100 mg/L MS-222 and then injected with 0.04 mL of vaccine.

After 21 days, corresponding to 536 degree/days, fish were challenged with 1.5×10^5 *S. iniae* cells/fish by an intracoelomic injection of 0.05 mL bacterial culture. Mortality was recorded for 12 days after challenge. During this period, dead fish were removed 3 times a day. Brain and kidney cultures of 70-80% of themoribund or dead fish were taken for microbiological testing to attempt to verify the cause of mortality.

Second experimental design. This experiment was designed to investigate whether or not a difference existed in the vaccine efficacy between adult RTB and rainbow sharks. Both RTB and rainbow shark adults were vaccinated with 0.1 mL of the oil (O) or aluminum (A) formulations. The control group was injected with 0.1 mL of sterile BHI.

Due to the size of the adult RTB and rainbow sharks, the fish were harvested from ponds and stocked in 700 L flow through vats. Due to available vats in the farm, only 3 vats for each treatment were used and stocked with 60 fish/vat. Fish were acclimatized for a week and then vaccinated.

One day before the challenge, corresponding to 21 days post-vaccination, fish were moved to the systems in our laboratory. Each tank was stocked with 12 fish. Four tanks were randomly assigned for each treatment and for the controls. Fish could not be challenged at the farm for biosecurity reasons, and because of the small volume of the tanks in our system (38 L) they could not be kept there for too long without stressing them. After 1 day of acclimation, RTB and rainbow adult sharks were challenged with 1.5×10^5 *S. iniae* cells/fish by an intracoelomic injection of 0.05 mL bacterial culture. Mortality was recorded for 12 days after challenge. During this period, dead fish were removed 3 times at day. Brain and kidney cultures of 70-80% of the moribund or dead fish were taken for microbiological testing in an attempt to verify the cause of mortality.

Histological samples. In the experiments with both juvenile and adult sharks, before the challenge and at the end of the experiment, 12 representative fish from each treatment were preserved in 10% formalin. Histological slides with hematoxylin-eosin or gram stain were prepared on a representative sample of 5 fish for each treatment.

Photomicrographs of the histological sections were captured on computer using the capture system Moticap L312 (Motic Instruments, Richmond, B.C., Canada) and a video camera Ultrak (Ultrak, Lewisville, Texas) connected to the computer and to a light microscope with 100x or 400x magnification.

Statistical analysis. ANOVA analysis and Tukey's post hoc test were run for each experiment using the statistical program SPSS 12.0 (SPSS Inc., Chicago, IL). An asin(square root) transformation was performed on the mortality data expressed as percentage. The results of the ANOVA analyses were considered significant at a $p\text{-value} < 0.01$; then the Tukey's test was considered at an $\alpha = 0.05$. Relative percent survival (RPS) was calculated with the formula:

$$\text{RPS} = \left[1 - \frac{\text{treatment mortality (\%)}}{\text{control mortality (\%)}} \right] * 100$$

12.3. Results

In the first experiment with juvenile fish, no mortalities were recorded in either rainbow or RTB sharks after vaccination, but 10% of the rainbow sharks injected with the aluminum formulation presented with ascites and hemorrhagic areas in the ventral part of the body and around the base of the pectoral and pelvic fins. The necropsy and bacterial cultures performed on four of these fish gave negative results for any bacterial or parasitic infections, and none of the remaining fish died before the challenge with *S. iniae*. None of the RTB sharks presented similar clinical signs.

From the results of the bacterial challenge of rainbow shark in the first experiment (Figure 12-1), no significant difference in mortality was observed among fish vaccinated with the basic, or the aluminum formulations administered by bath, fish vaccinated with

the basic formulation by injection, and the control group ($100 \pm 0\%$ mortality in all treatments). Significantly lower mortality was observed only in fish vaccinated by injection with the aluminum or oil formulations ($92 \pm 7\%$ and $89 \pm 9\%$ mortality respectively for the aluminum and oil formulation). Significantly lower mortality was observed in RTB shark vaccinated by injection with the aluminum formulation ($65 \pm 2\%$ mortality) compared to the control fish ($100 \pm 0\%$).

In the experiment with adult rainbow and RTB sharks (Figure 12-2 and 12-3), the mortalities of fish vaccinated with the aluminum and oil formulations were significantly lower than the controls ($18 \pm 18\%$ and $18 \pm 23\%$ mortality in RTB shark, $35 \pm 5\%$ and $75 \pm 6\%$ mortality in rainbow shark respectively for the aluminum and oil formulation). In rainbow shark the mortality in fish vaccinated with the aluminum formulation was significantly lower than in fish vaccinated with the oil formulation; while in RTB shark, no significant difference was observed between the two vaccines.

Examination of histological sections of vaccinated juvenile and adult RTB sharks revealed the presence of large and numerous granuloma-like lesions in the spleen, liver, anterior and posterior kidney. In juvenile and adult rainbow sharks similar lesions were seen only in the spleen.

12.4. Discussion

The results of the bacterial challenge in the first experiment showed that neither the basic nor the aluminum formulations administered by bath provided significant protection for the rainbow shark compared to the control group; only administration by injection of the vaccine formulations with the adjuvant was slightly effective in producing a protection level in the fish, the basic formulation was not effective. Injection

of the aluminum formulation gave a significantly higher level of protection in RTB shark than in the rainbow shark.

In the experiment with adult rainbow and RTB sharks, both the aluminum and oil formulations were effective in protecting the fish compared to the control groups; and both vaccine formulations gave better results in RTB shark than in rainbow shark. In rainbow shark the aluminum formulation gave a significantly higher level of protection than the oil formulation; however, in RTB shark no difference was observed between the two vaccines.

The difference in vaccine efficacy between the rainbow and red-tail black sharks is one of the more interesting results of this research. It is well known that in farmed animals and plants, strains exist that are more resistant to disease. Similar considerations are also valid for fish. Several studies in fish have investigated the possibility of using lysozyme activity as an immune parameter for indirect selection of more resistant strains of fish (Lund et al. 1995; Roeed et al. 1993) and in Atlantic salmon (*Salmo salar*) it was found that a significant genetic variation in lysozyme activity was inversely associated with high rates of survival (Lund et al. 1995). In other studies it was found that a correlation exists between disease resistance and genetic variation in antibody concentration (Fjalestad et al. 1996; Stroemsheim et al. 1994). Genetic variations in resistance to the viral haemorrhagic septicemia virus (VHSV) were observed in rainbow trout (*Oncorhynchus mykiss*) (Dorson and Torhy, 1994; Dorson et al. 1995) and virus-resistant strains of fish were selected for breeding. Similar research was conducted using lines and their hybrids of common carp (*Cyprinus carpio*) to examine genetic differences in resistance against *Aeromonas salmonicida* (Wiegertjes et al. 1995a) and

Trypanoplasma borreli (Wiegertjes et al. 1995b). Genetic variations in resistance to *Cryptobia salmositica* (Chin et al. 2004) and to *Gyrodactylus salaris* (Dalgaard et al. 2004) were observed among populations of Atlantic salmon. Interspecific genetic variations in resistance to the VHSV, infectious haematopoietic necrosis virus (IHNV), and infectious pancreatic necrosis virus (IPNV) were also observed among rainbow trout, arctic char (*Salvelinus alpinus*), and lake trout (*S. namaycush*) and their hybrids (Dorson et al. 1991).

In this research we created an acceptable model to investigate the different immunostimulatory effects of vaccines comparing juveniles and adults of both ornamental shark species. Interestingly, the different immunostimulatory effect of the vaccines could be observed both in the histological analyses of the fish immune organs and in the results of the challenge tests. The histological analyses of the effect of the oil and aluminum vaccines on the immune organs confirmed the observations made in previous studies with RTB shark (Chapter 11) as well as the hypothesis that the granuloma-like lesions stimulated by the aluminum formulation may be correlated with the protection levels. In fact in RTB sharks where the vaccine conferred the highest protection level, granulomas were observed in spleen, posterior kidney and liver. In contrast, in the rainbow sharks, granulomas were observed only in the spleen.

Differences between the two species were also observed in the experiments run during development of the challenge model (Chapter 9). The *S. iniae* infection developed faster in the rainbow shark population, and deaths were no longer observed 4 days after the bacterial challenge. In contrast, deaths were still observed 11 days post-challenge in the RTB shark. Furthermore, the mortality rates were almost always higher in rainbow

than in RTB shark injected with the same concentration of bacteria. Differences between the two species were also observed with respect to the presumed toxic side effects of the aluminum formulation injection (Chapter 10). Mortality but no external signs were observed in RTB shark injected with 0.06 mL of vaccine; however, injection with 0.04 mL caused no side effects. In 10% of the rainbow sharks, dropsy and hemorrhagic areas on the ventral part of the body and at the base of the pelvic and pectoral fins were observed when fish were injected with 0.04 mL of the aluminum formulation.

The results of this research suggest that there may be a difference in the immunostimulatory effect between juvenile and adult shark of both species (see Table 12-1), however, more experiments are needed to confirm this hypothesis.

The protection levels conferred by the vaccine we tested in sharks are similar to the results observed in research with other species of fish such as yellowtail (Iida et al. 1982; Kusuda and Takagi, 1983), tilapia (Bercovier et al. 1997; Eldar et al. 1995b; Klesius et al. 2000; Klesius et al. 2001), and rainbow trout (Eldar et al. 1997; Hortvitz et al. 1997; Sakai et al. 1987).

12.5. Conclusions

This research demonstrated that the immunostimulatory effect of vaccines might be significantly different among closely related species and between juveniles and adults. For this reason, before beginning a large scale vaccination procedure with other species, it is important to conduct pilot trials to determine if the same vaccine is effective in other species. However, it may also be possible that the vaccine is more effective in other species than the target species.

Disease resistance due to genetic variation, as observed in this research, is an important factor that should be considered by every aquaculture facility to increase productivity and decrease production expenses. To develop an effective genetic improvement program, it is important to consider several selection criteria, such as survival, environmental adaptation, growth, reproduction, and several factors such as inheritance, genetic correlations, and repetition. Breeding fish with a more responsive immune system may reduce mortality from disease outbreaks, improve the efficacy of vaccinations, and decrease the application of chemotherapeutics.

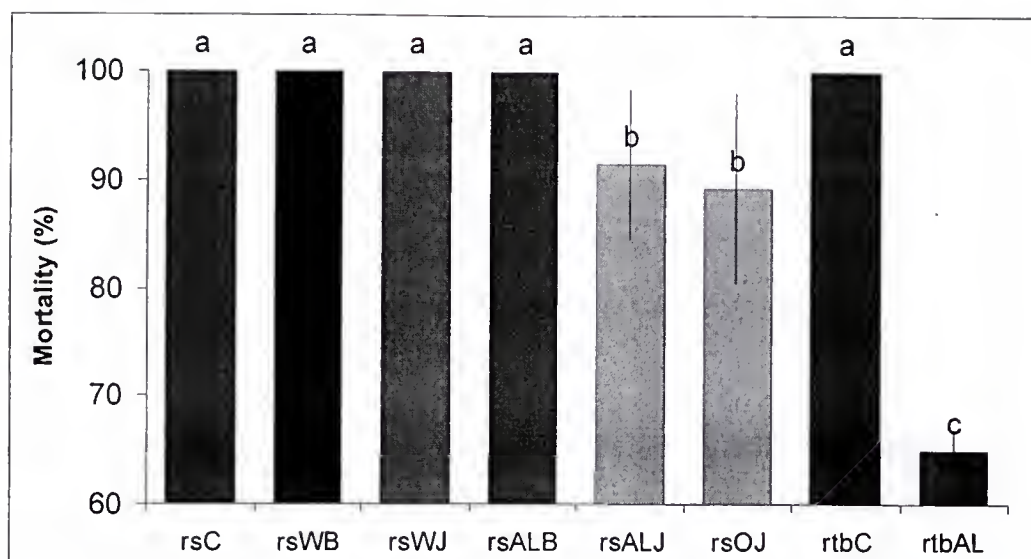


Figure 12-1. Rainbow and RTB juvenile sharks average mortality (%) recorded for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish). Bars represent the standard deviation; letters above the bars represent the level of the significant difference among treatments as resulted from the Tukey's test ($p = 0.0045$, $\alpha = 0.05$); 4 replicates were used per treatment and each tank was stocked with 25 fish. Abbreviations: rs= rainbow shark, rtb= RTB shark, C= control, WB= basic formulation by bath, ALB aluminum formulation by bath, WJ= basic formulation by injection, ALJ= aluminum formulation by injection, OJ= oil formulation by injection.

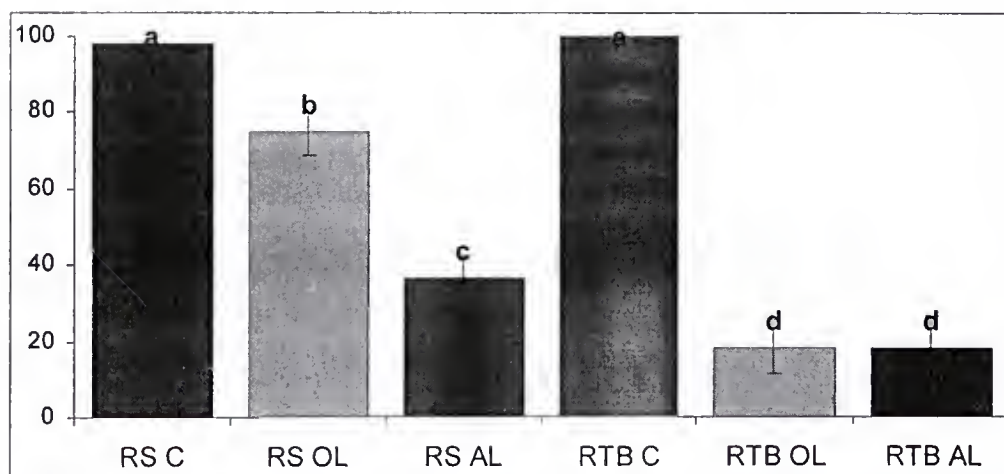


Figure 12-2. Rainbow and RTB adult sharks average mortality (%) recorded for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish). Bars represent the standard deviation; letters above the bars represent the level of the significant difference among treatments based on Tukey's test ($p = 6.5 \times 10^{-7}$, $\alpha = 0.05$); 4 replicates were used per treatment and each tank was stocked with 12 fish. Abbreviations: RS= rainbow shark, RTB= RTB shark, C= control, AL= aluminum formulation by injection, OL= oil formulation by injection.

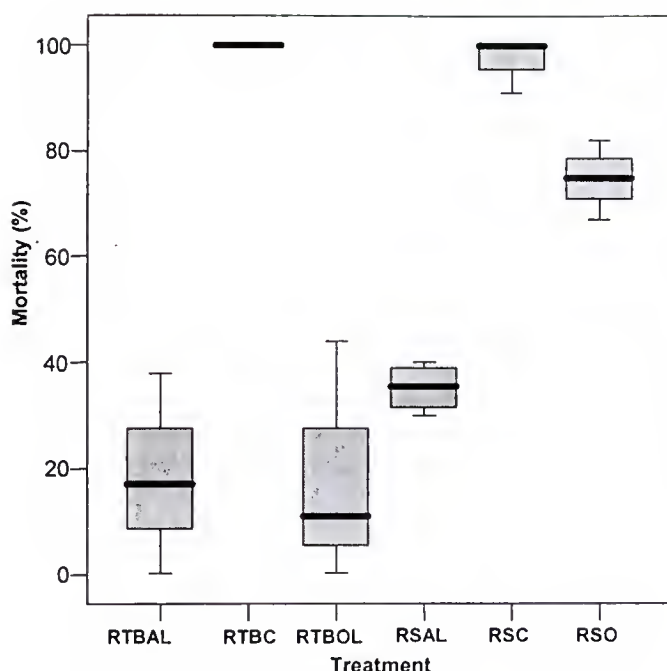


Figure 12-3. Box plot of the rainbow and RTB adult sharks average mortality (%) recorded for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish). Boxes and bars respectively represent the distribution of 50% and 100% of data. The box plots gave an easier visualization of the range of values of each treatment; 4 replicates were used per treatment and each tank was stocked with 12 fish. Abbreviations: RS= rainbow shark, RTB= RTB shark, C= control, AL= aluminum formulation by injection, OL= oil formulation by injection.

Table 12-1. Total average mortality (%) recorded in RTB and rainbow sharks juveniles and adults for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish).

Species	Age	Treatment	Mortality %±SD
RTB shark (<i>E. bicolor</i>)	juvenile	C	100 ± 0
		AL	65 ± 2
	adult	C	100 ± 0
		AL	18 ± 18
		OL	18 ± 23
Rainbow shark (<i>E. erythrurus</i>)	juvenile	C	100 ± 0
		AL	92 ± 7
		OL	89 ± 9
	adult	C	98 ± 2
		AL	35 ± 5
		OL	75 ± 6

Abbreviations: AL= aluminum formulation by injection, OL= oil formulation by injection; 4 replicates were used per treatment and each tank was stocked with 25 juvenile or 12 adult fish.

CHAPTER 13
DIFFERENCE IN RESISTANCE TO *Streptococcus iniae* INFECTIONS BETWEEN
THE WILD PHENOTYPIC RAINBOW SHARK (*Epalzeorhynchus erythrurus*, FAM.
CYPRINIDAE) AND AN ARTIFICIALLY SELECTED PHENOTYPE (THE BLUE
SHARK)

13.1. Introduction

Disease resistance, or disease-tolerance, and immune responses are under polygenic control and genetic variations to many diseases and immunological parameters have been established in fish (Chevassus and Dorson, 1990; Chin et al. 2004; Dorson et al. 1991; Fjalestad et al. 1993; Gjedrem et al. 1991; Gjedrem and Gjoen, 1995; Wieger et al. 1996). It is known from studies in mammals that the major histocompatibility complex (MHC) is involved in the regulation of the immune response and is responsible for differences in disease resistance among individuals of the same species. Genes related to MHC class I and class II have been sequenced and described in salmonids (Knight et al. 1998; Shum et al. 2001), in carp, zebrafish, and a number of other cyprinid and non-cyprinid species (Stet et al. 1998).

The adaptive immune system is also involved in generating long-term protection, and for this reason adaptive immunological parameters are valid candidates as indirect markers for resistance. Several studies in fish have investigated the possibility of using lysozyme activity as an immune parameter for indirect selection of more resistant strains of fish (Lund et al. 1995; Roed et al. 1993), and in Atlantic salmon (*Salmo salar*) it was found that a significant genetic variation in lysozyme activity was inversely associated with high survival rates (Lund et al. 1995). A significant correlation between survival and

genetic variation in antibody titers was found in Atlantic salmon with antibodies against *Aeromonas salmonicida* (Stroemsheim et al. 1994), and with antibodies against *Vibrio salmonicida* and *V. anguillarum* (Fjalestad et al. 1996). A correlation between genetic susceptibility and humoral response to infectious hematopoietic necrosis virus (IHNV) was also observed in brown trout (*Salmo trutta*) and lake trout (*Salvelinus namaycush*) (LaPatra et al. 1996).

In earlier studies, we observed differences in the streptococcal infection and in the vaccine efficacy between red-tail black shark (RTB shark) and rainbow shark. In this study we wanted to investigate the possibility of different susceptibility to *S. iniae* between rainbow and blue sharks. Blue shark have been artificially selected by farmers from rainbow shark by repetitive inbreeding of these variants. Rainbow sharks are brownish with red fins, whereas blue sharks have blue body coloration and white fins. Farmers have reported that blue sharks are much more resistant to *S. iniae* than rainbow sharks and, in order to examine this hypothesis, an investigation was conducted.

13.2. Materials and Methods

Experimental animals and systems. The research was conducted at the Tropical Aquaculture Laboratory, University of Florida (Ruskin, FL) using two recirculating systems with 25 tanks of 38-L volume. Blue (1.4 ± 0.2 g WT, 5.2 ± 0.6 TL), rainbow (1.1 ± 0.3 g WT, 4.9 ± 0.3 TL) and RTB shark juveniles (1.6 ± 0.5 g WT, 5.5 ± 0.6 TL) were obtained from an ornamental fish farm in Hillsborough county (FL). A representative group of 30 individuals for species was measured at the beginning of the research study. To verify the *S. iniae*-free status of the fish, brain and kidney tissues were sampled from 5 representative selected fish before each experiment. The samples were

spread on Tryptic Soy Agar with 5% sheep's blood (Physician's Laboratory Supply, Troy, Michigan) and incubated at 37°C for 48 h.

Temperature, ammonia, and nitrite in the water systems were measured daily; alkalinity, hardness and pH once at week. The water in the system was maintained at $25.5 \pm 0.6^\circ\text{C}$. Water ammonia and nitrite concentrations were always close to 0 mg/L. Alkalinity and hardness were 110 ± 10 mg/L and 115 ± 15 mg/L respectively; pH was 7.5.

Bacterial strain. Details of the bacterial isolation and batch broth culture were reported previously (Chapter 9).

Vaccine production. Details of the vaccines production were reported previously (Chapter 10). In these experiments only the aluminum formulation was used.

First experimental design. This experiment was designed to investigate whether or not a difference existed in the vaccine efficacy among RTB, rainbow and blue shark juveniles. Fish were vaccinated by intracoelomic injection of 0.04 mL of aluminum vaccine formulation; the control group for each species was injected with 0.04 mL of sterile BHI.

Juvenile sharks of each species were stocked at a density of 20 fish/tank and four tanks were randomly assigned for each treatment and for the controls. Fish were vaccinated after a week of acclimation. Groups of 10 fish were anesthetized with 100 mg/L MS-222 and then injected with 0.04 mL of vaccine.

After 21 days, corresponding to 536 degree/days, fish were challenged with 1.5×10^5 *S. iniae* cells/fish by an intracoelomic injection of 0.05 mL bacterial culture. Mortality was recorded for 12 days after challenge. During this period, dead fish were

removed 3 times at day. Brain and kidney cultures of 70-80% of the moribund or dead fish were taken for microbiological testing to attempt to verify the cause of mortality.

Second experimental design. In this experiment the same protocol used in the previous experiment was followed, except that in this study 3 replicates were used for each treatment, and fish were challenged with 3×10^5 *S. iniae* cells/fish.

Statistical analysis. ANOVA analysis and Tukey's post hoc test were run for each experiment using the statistical program SPSS 12.0 (SPSS Inc., Chicago, IL). An asin(square root) transformation was performed on the mortality data expressed as percentage. The results of the ANOVA analyses were considered significant at a $p\text{-value} < 0.01$; then the Tukey's test was considered at an $\alpha = 0.05$. Relative percent survival (RPS) was calculated with the formula:

$$\text{RPS} = \left[1 - \frac{\text{treatment mortality (\%)}}{\text{control mortality (\%)}} \right] * 100$$

13.3. Results

In the first experiment (Figure 13-1, Table 13-1) there was no difference in mortality ($100 \pm 0\%$) between the vaccinated and the control rainbow sharks. The mortality of vaccinated RTB shark ($69 \pm 7\%$) was significantly lower than the mortality of the control group ($87 \pm 2\%$). In blue shark, no significantly difference in average mortality was observed between vaccinated ($78 \pm 8\%$) and control fish ($77 \pm 8\%$).

Similar results for rainbow and RTB sharks were observed in the second experiment (Figure 13-2, Table 13-1), but, in this case, there was a significantly difference in average mortality at the end of the experiment between vaccinated ($90 \pm 8\%$) and control blue sharks ($100 \pm 0\%$).

13.4. Discussion

The results of this study demonstrated that there may be an intraspecific difference in susceptibility to *S. iniae* between rainbow shark and the artificially selected variant blue sharks, with blue shark being more resistant than rainbow shark. Interestingly, blue sharks appear to be as resistant to *S. iniae* as RTB sharks. An interspecific difference was observed in the vaccine efficacy. The aluminum vaccine was effective in protectioning RTB shark, but gave no protection to rainbow sharks. Similar results were observed in a previous study (Chapter 12). It also appears that in blue shark the vaccine is not very effective, as observed by the absence of conferred protection in the first experiment, and by the small decrease (10%) in mortality observed in the second experiment.

It is well known that there are animals and plant strains that are more resistant to specific disease, but an interesting result of this research is that this difference is also associated with a different color pattern /phenotype in the rainbow shark. Genetic variations in resistance among populations of the same species and heritability of this resistance characters have been observed in rainbow trout (*Oncorhynchus mykiss*) against viral haemorrhagic septicemia virus (VHSV) (Dorson and Torhy, 1994; Dorson et al. 1995), in common carp (*Cyprinus carpio*) against *Aeromonas salmonicida* (Wiegertjes et al. 1995a) and against *Trypanoplasma borreli* (Wiegertjes et al. 1995b), in Atlantic salmon against *Cryptobia salmositica* (Chin et al. 2004), against *Gyrodactylus salaris* (Dalgaard et al. 2004) and against *V. salmonicida* (Lund et al. 1995). Interspecific genetic variations in resistance to the VHSV, infectious haematopoietic necrosis virus (IHNV), and infectious pancreatic necrosis virus (IPNV) were also observed among rainbow trout,

arctic char (*Salvelinus alpinus*), lake trout (*S. namaycush*), and their hybrids (Dorson et al. 1991).

13.5. Conclusions

This study demonstrated that differences in resistance to disease and differences in vaccine efficacy could be detected in the same species of fish among different phenotypes from the same population, and among closely related species. Disease resistance due to genetic variation is an important factor that should be considered for developing successful breeding programs. The selection of strains of fish less susceptible to disease results in an increase of production and a decrease in production expenses, represented for example by decreased mortality and drug applications.

The different results of vaccine efficacy in blue sharks observed between the two experiments gives an example of the different possible outcomes of vaccination. Especially in the field, there is often a misunderstanding as to what is meant by “vaccine efficacy” and the fact that the results of vaccination procedures can be uncertain. Vaccines help to prevent diseases and reduce disease severity, but do not prevent infections. The effect of vaccination is usually observed over a long period of time and consists in decreasing or eliminating disease incidence. For this reason vaccine efficacy is more complicated to predict than disease elimination. Vaccines can also increase resistance to other disease or stressful situations, improved growth rate and feed efficiency so as to make their use economically effective even if there is no decrease in mortality (Mitchell, 1997; Mitchell, 1999). The efficacy of vaccines is also influenced by many factors (genetic, nutrition quality, stress, water temperature, handling), and most of them are either uncontrollable or underestimated. For this reason, although great

differences in results might be seen among different facilities and it is not uncommon that a group of vaccinated fish shows the same mortality rate as control fish, this does not necessarily imply that a vaccination program is not efficacious (Horne and Robertson, 1987).

Table 13-1. Total average mortality (%) recorded in RTB, rainbow and blue sharks for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish). In the last column the level of the significant difference among treatments in each experiment based on Tukey's tests are reported.

Experiment	Treatment	Average Mortality %±SD	Significance
1	RTBC	87 ± 2	b
	RTBV	69 ± 7	a
	RSC	100 ± 0	c
	RSV	100 ± 0	c
	BLC	77 ± 8	a,b
	BLV	78 ± 8	a,b
2	RTBC	100 ± 0	d
	RTBV	86 ± 6	e
	RSC	100 ± 0	d
	RSV	100 ± 0	d
	BLC	100 ± 0	d
	BLV	90 ± 8	e

($p_1 = 4.2 \times 10^{-9}$, $p_2 = 9.0 \times 10^{-5}$, $\alpha = 0.05$); in the first experiment 4 replicates were used per treatment and in the second experiment 3 replicates, in both experiments each tank was stocked with 20 fish. Abbreviations: BL= blue shark, RS= rainbow shark, RTB= RTB shark, C= control, V= vaccinated fish.

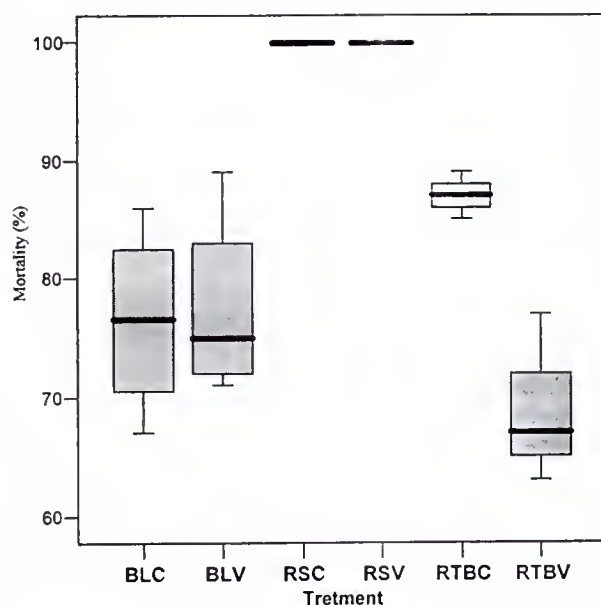


Figure 13-1. Box plot of average mortality (%) recorded in the first experiment with RTB, rainbow and blue sharks for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish); 4 replicates were used per treatment and each tank was stocked with 20 fish. Boxes and bars respectively represent the distribution of 50% and 100% of data. Abbreviations: BL= blue shark, RS= rainbow shark, RTB= RTB shark, C= control, V= vaccinated fish.

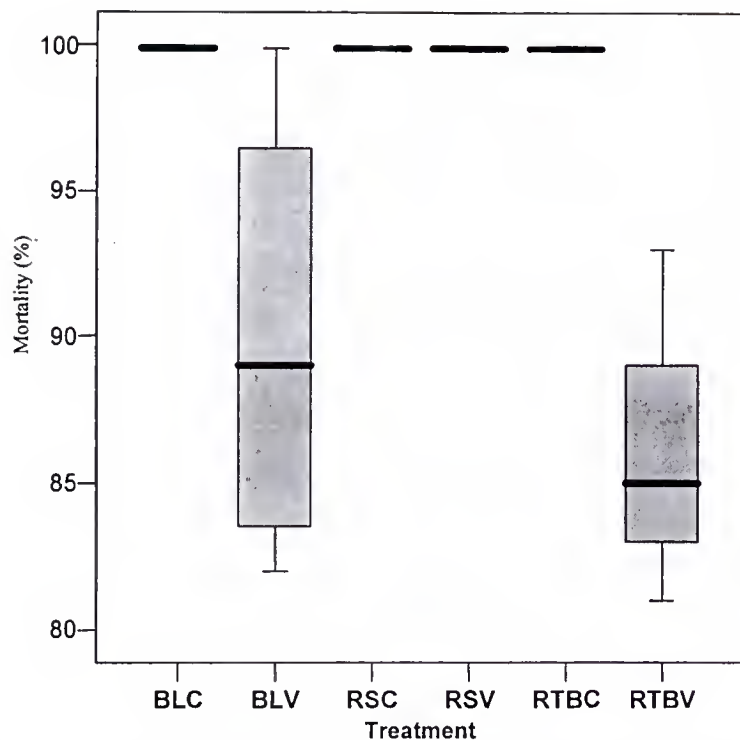


Figure 13-2. Box plot of average mortality (%) recorded in the second experiment with RTB, rainbow and blue sharks for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish); 3 replicates were used per treatment and each tank was stocked with 20 fish. Boxes and bars respectively represent the distribution of 50% and 100% of data. Abbreviations: BL= blue shark, RS= rainbow shark, RTB= RTB shark, C= control, V= vaccinated fish.

CHAPTER 14
ENHANCEMENT OF RESISTANCE TO *Streptococcus iniae* INFECTIONS IN
VACCINATED AND UNVACCINATED RED-TAIL BLACK SHARK
(*Epalzeorhynchus bicolor*, FAM. CYPRINIDAE) FED BETA-GLUCANS OR
NUCLEOTIDES

14.1. Introduction

Recent studies, especially in the field of human medicine, have been investigating the immunostimulatory effects of nucleotides (Van Buren et al. 1994). Dietary nucleotides promote growth, maturation of the developing gut, regeneration, healing of the intestinal mucosa and increased resistance to disease (Carver, 1994; Kulkarni et al. 1986; McCauley et al. 1998). This increase in resistance to infection is reported to be a consequence of increasing phagocytic activity of peritoneal macrophages (Kulkarni et al. 1986), T-cell dependent antibody production (Jyonouchi, 1994), natural killer cell (NK) activity (Carver, 1994), interleukin-2 production (Carver, 1994), and bone marrow cell and peripheral neutrophil numbers (Matsumoto et al. 1995).

Beta-1-3 and beta-1-6 glucans are derived from yeast and fungi. Differences in the immunostimulatory effect of glucans may be the result of their source, isolation procedures, number, length, and relative portion of beta-1-3- and beta-1-6- linked side chains (Raa, 1996). Glucans have been shown to increase macrophage chemotactic and phagocytic activity as well as lysozyme and complement activity; to stimulate cytokine (IL-1, TNF) production, and leukocyte migration through macrophage activation (Secombes and Fletcher, 1992); and to significantly reduce mortality after fish were

challenged with various pathogens (Abel and Czop, 1992; Sealey and Gatlin, 1999; Verlhac et al. 1996; Verlhac et al. 1998).

The purpose of the following studies was to test the effectiveness of beta-glucans and nucleotides for increasing the survival of vaccinated and nonvaccinated red-tail black shark (RTB shark) after challenge with *Streptococcus iniae*.

14.2. Materials and Methods

Experimental animals and systems. The research was conducted at the Tropical Aquaculture Laboratory, University of Florida (Ruskin, FL). Two experiments were conducted using two recirculating systems with 25 tanks of 38-L volume. RTB shark juveniles (1.4 ± 0.4 g WT, 5.6 ± 0.5 TL) were obtained from an ornamental fish farm in Hillsborough county (FL). A representative group of 30 individuals for each species was measured at the beginning of the research. The farms sell sharks when they reach a size of 2.5-5 cm, and for this reason, farmers are interested in vaccinating mainly juveniles. For this reason we chose to use fish of this size in our research. To verify the *S. iniae*-free status of the fish, brain and kidney tissues were sampled from 5 representative fish before each experiment. The samples were spread on Tryptic Soy Agar with 5% sheep's blood (Physician's Laboratory Supply, Troy, Michigan) and incubated at 37°C for 48 h.

Water temperature, ammonia, and nitrite in the systems were measured daily; alkalinity, hardness and pH once at week. The water in the system was maintained at $25.5 \pm 0.6^\circ\text{C}$. Water ammonia and nitrite concentrations were always close to 0 mg/L. Alkalinity and hardness were 110 ± 10 mg/L and 115 ± 15 mg/L respectively; pH was 7.5.

Bacterial strain. Details of the bacterial isolation and batch broth culture were reported previously (Chapter 9).

Vaccine production. Details of the vaccines production were reported previously (Chapter 10). In these experiments only the aluminum formulation was used.

First experimental design. This experiment was designed to investigate whether or not a difference existed in the vaccine efficacy among vaccinated and nonvaccinated RTB shark fed nucleotides, or beta-glucans, or a control diet. Beta-glucans (Macrogard™, Tromso, Norway) and nucleotides (AQUAGENT™, NOVARTIS-Aqua Health LTD., Charlottetown, Canada) were added to a formulated diet (48 % protein, 10.7 % fat, 5 % fiber) formulated without any yeast source (Laramore and Hopkins Inc., Fort Pierce, Florida) following the recommended doses, respectively of 1g/ kg feed and 2g/ kg feed, and fed twice each day at 3% body weight. Diets with immunostimulants were fed for 24 days following the fourth day after stocking the fish in the systems. Fish were vaccinated by intracoelomic injection of 0.04 mL of the aluminum vaccine formulation; the control group was injected with 0.04 mL of sterile BHI.

Each tank was stocked with 25 RTB sharks, and eight tanks were randomly assigned to each treatment and to the controls. Fish were vaccinated after a week of acclimation. Groups of 10 fish were anesthetized with 100 mg/L MS-222 and then injected with 0.04 mL of vaccine.

After 21 days, corresponding to 536 degree/days, fish were challenged with 1.5×10^5 *S. iniae* cells/fish by an intracoelomic injection of 0.05 mL of bacterial culture. Mortality was recorded for 12 days after challenge. During this period, dead fish were

removed 3 times at day. Brain and kidney cultures of 70-80% of the moribund or dead fish were taken for microbiological testing to attempt to verify the cause of mortality.

Second experimental design. In this experiment the same protocol used in the previous experiment was followed, except that 4 replicates were used for each treatment, and beta-glucans and nucleotides were fed only to vaccinated fish.

Statistical analysis. ANOVA analysis and Tukey's post hoc test were run for each experiment using the statistical program SPSS 12.0 (SPSS Inc., Chicago, IL). An asin(square root) transformation was performed on the mortality data expressed as percentage. The results of the ANOVA analyses were considered significant at a $p\text{-value} < 0.01$; then the Tukey's test was considered at an $\alpha = 0.05$. Relative percent survival (RPS) was calculated with the formula:

$$\text{RPS} = \left[1 - \frac{\text{treatment mortality (\%)}}{\text{control mortality (\%)}} \right] * 100$$

14.3. Results

In the first experiment (Table 14-1, Figure 14-1 and 14-2) the mortality of both vaccinated and nonvaccinated fish fed beta-glucans ($23 \pm 7\%$ and $82 \pm 1\%$ respectively) or nucleotides ($28 \pm 6\%$ and $86 \pm 5\%$ respectively) was significantly lower than the mortality of the control group ($35 \pm 4\%$ and $93 \pm 5\%$ respectively), but there was no significant difference in mortality between fish fed beta-glucans or nucleotides.

Also in the second experiment (Figure 14-3) the mortality of vaccinated fish fed beta-glucans ($25 \pm 7\%$) or nucleotides ($43 \pm 9\%$) was significantly lower than the control group ($69 \pm 7\%$). In this experiment the mortality of fish fed beta-glucans was significantly lower than fish fed nucleotides.

14.4. Discussion

The results of this research demonstrated that both beta-glucans and nucleotides are very efficient in stimulating the immune system of RTB shark and in decreasing mortality by at least 10% after bacterial challenge. The results of the first experiment demonstrated that both nucleotides and beta-glucans are efficient in decreasing the mortality of fish challenged with *S. iniae*, independently of whether the fish were vaccinated or not. The results of the second experiment confirmed the protective effect given by both immunostimulants. In the first experiment beta-glucans appeared to give better protection, although this was not significantly different than nucleotides: on average, the beta-glucans decreased the mortality by 11.5%, the nucleotides by 7%. In the second experiment beta-glucans conferred a significantly higher protection than nucleotides, decreasing on average the mortality of 44% versus 26 % for the nucleotides.

In a pilot trial run with 24 vaccinated fish fed beta-glucans or nucleotides and 24 vaccinated fish from the control group (all surviving fish from the first experiment), we observed that the mortality of fish fed immunostimulants and challenged with *S. iniae* was 43% compared to 88% for the control fish (Table 1). Based on these preliminary results, the protection conferred by the immunostimulants may last at least for more than 3 months after stopping their administration. However, these results are only indicative because no treatment replicates were used due to the low number of available fish.

As seen in the first experiment, the level of conferred protection provided by beta-glucans or nucleotides was not different in vaccinated and unvaccinated fish. This result might be explained by the hypothetical mechanisms of action of these compounds, believed to stimulate the non-specific portion of the immune system more than the

specific portion. It has been shown that nucleotides and especially beta-glucans stimulate natural killer cell (NK) and macrophage activity (Carver, 1994; Kulkarni et al. 1986; Secombes and Fletcher, 1992), although both compounds can also enhance antibody production through T-cell action (Jyonouchi, 1994; Nikl et al. 1993; Rorstad et al. 1993; Verlhac et al. 1996).

The decreased mortality we observed in fish fed beta-glucans is in accordance with the results observed in other studies. Lower mortality was observed in Atlantic salmon vaccinated against *Aeromonas salmonicida*, (Nikl et al. 1993; Rorstad et al. 1993), in turbot vaccinated against *Enterococcus* sp. (Toranzo et al. 1995), in yellowtail challenged with *Enterococcus seriolicida* (Itami et al. 1996), and in swordtails, rosy barbs, black tetras challenged with *A. hydrophila* or *Pseudomonas fluorescens* (Tuernau et al. 2000) fed beta-glucans. Glucans from lentinan, schizophyllan, and scleroglucan increased the survival rate of carp challenged with *Edwardsiella tarda* (Yano et al. 1991). However, in some cases an enhancement of the nonspecific immune response rather than survival was observed in fish fed glucans, as in catfish challenged with *Edwardsiella ictaluri* (Duncan and Klesius, 1996), and in turbot challenged with *Vibrio anguillarum* (de Baulny et al. 1996). In hybrid bass infected with *S. iniae*, glucans showed limited effects (Sealey and Gatlin, 2002).

Many studies have been conducted in mammals to investigate the effects of nucleotides on the immune system (Carver, 1994; Jyonouchi, 1994; Kulkarni et al. 1986; Matsumoto et al. 1995; McCauley et al. 1998; Van Buren et al. 1994), and it is known that the mortality of mice fed nucleotides and challenged with *Staphylococcus aureus* (Kulkarni et al. 1986) or *Candida albicans* (Carver, 1994) is lower than mice fed

nucleotide-free diets. However, there is very limited research on the effect of dietary nucleotides on fish. In our experiments nucleotides successfully decreased the mortality of fish challenged with *S. iniae*. Similar results were observed in hybrid bass challenged with *S. iniae* (Gatlin et al. 2004), in rainbow trout challenged with *V. anguillarum*, in coho salmon challenged with *Piscirickettsia salmonis*, and in Atlantic salmon challenged with Infectious Salmon Anaemia Virus (ISAV) (Burrells et al. 2001), or *A. salmonicida* (Burrells, unpublished data). Most recent studies in mammals have shown that intraperitoneal administration of nucleotides may give better results than the use of nucleotide supplements in the diet due to the partial degradation of orally administered nucleotides in the gastrointestinal tract (Veerabagu et al. 1996).

One interesting finding of our experiments is that there was not a constant protection level conferred by the vaccine and the immunostimulants between the two reported experiments. These results demonstrated the different possible outcomes of vaccination, in this case extending to the use of immunostimulants. There is often a misunderstanding in the field of what is meant by vaccine efficacy and the results of vaccination procedures can be uncertain. As discussed previously, vaccines help prevent diseases and reduce outbreak severity, but are not intended to eliminate infections. The effects of vaccination occur over a long period of time and these effects are more complicated to measure than disease elimination. Furthermore, it is even more difficult to measure the real efficacy of the vaccine in the field because of the lowered incidence or absence of disease, which show their effect. Vaccines can increase resistance to other disease or stressful situations, and improve the growth rate and feed efficiency, thereby making their use economically effective even if there is no real decrease in mortality

(Mitchell, 1997; Mitchell, 1999). The efficiency of vaccines is influenced by many factors (genetic, nutrition quality, stress, water temperature, handling), and most of them are uncontrollable or underestimated. For this reason, considerable differences in results may be seen among facilities and it is not uncommon that a group of vaccinated fish shows the same mortality rate as that of control fish without proving that vaccines are not efficacious (Horne and Robertson, 1987).

14.5. Conclusions

These studies support the hypothesis that beta-glucans and nucleotides can help in decreasing the mortality of fish challenged with bacteria. Producers can use them cheaply and easily. These compounds can be supplemented at concentrations of a few grams per kg of feed, and fed for cycles of 3-4 weeks providing protection that may last for several months.

In previous experiments we showed the efficacy of the vaccine against *S. iniae* for rainbow and RTB sharks, but unfortunately only the injection route proved effective. It would not be very practical to vaccinate juvenile fish (2-5 cm TL), but the results of these experiments suggest that immunostimulants may have value if orally administered to juvenile fish. Future experiments should determine an optimal administration protocol, because, as seen in other studies, the body may produce a homeostatic response after prolonged administration of any immunostimulant, with consequent elimination of any immunostimulatory effects (Ortuno et al. 1999).

Table 14-1. Total average mortality (%) recorded in RTB shark fed immunostimulants or immunostimulants free-diets for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish) in the two experiments and in survival fish challenged 3 months after vaccination. In the last column the level of the significant difference among treatments as resulted from the Tukey's test for each experiment are reported.

Experiment	Treatment	Average Mortality %±SD	RPS %	Significance
1	C	93 ± 5	0	a
	N	86 ± 5	8	b
	B	82 ± 1	12	b
	CV	35 ± 4	62	c
	NV	28 ± 6	70	c,d
	BV	23 ± 7	75	d
2	C	87 ± 2	0	e
	CV	69 ± 7	21	f
	NV	43 ± 9	51	g
	BV	25 ± 7	71	h
3	CV	88 ± 6	0	
	BNV	43 ± 4	51	

($p_1 = 7.5 \times 10^{-29}$, $p_2 = 4.5 \times 10^{-4}$, $\alpha = 0.05$); 8 replicates were used per treatment in the first experiment and 4 replicates in the second experiment, in both experiments each tank was stocked with 25 fish. Abbreviations: RPS= relative percent survival, C= fish fed control diet, N= fish fed nucleotides but not vaccinated, B= fish fed beta-glucans but not vaccinated, V= vaccinated fish.

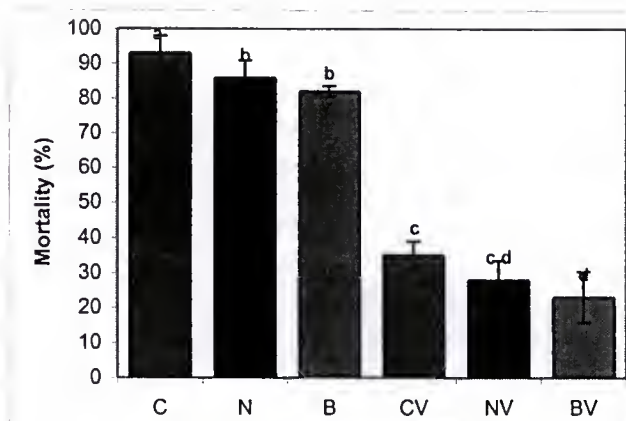


Figure 14-1. Average mortality rate (%) recorded in the first experiment with RTB shark fed immunostimulants and immunostimulants free-diets for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish). Bars represent the standard deviation; letters above the bars represent the level of the significant difference among treatments based on a Tukey's test; 8 replicates were used per treatment and each tank was stocked with 25 fish. $p_2 = 4.5 \times 10^{-4}$, $\alpha = 0.05$. Abbreviations: C= fish fed control diet, N= fish fed nucleotides but not vaccinated, B= fish fed beta-glucans but not vaccinated, V= vaccinated fish.

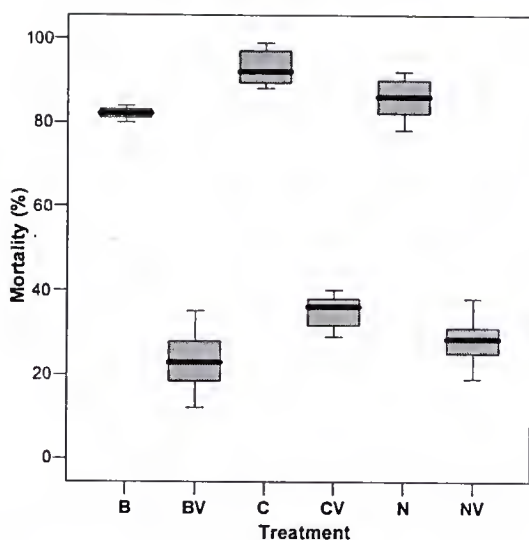


Figure 14-2. Average mortality rate (%) recorded in the first experiment with RTB shark fed immunostimulants and immunostimulants free-diets for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish); 8 replicates were used per treatment and each tank was stocked with 25 fish. Boxes and bars respectively represent the distribution of 50% and 100% of data. The box plots gave an easier visualization of the range values of each treatment. Abbreviations: C= fish fed control diet, N= fish fed nucleotides but not vaccinated, B= fish fed beta-glucans but not vaccinated, V= vaccinated fish.

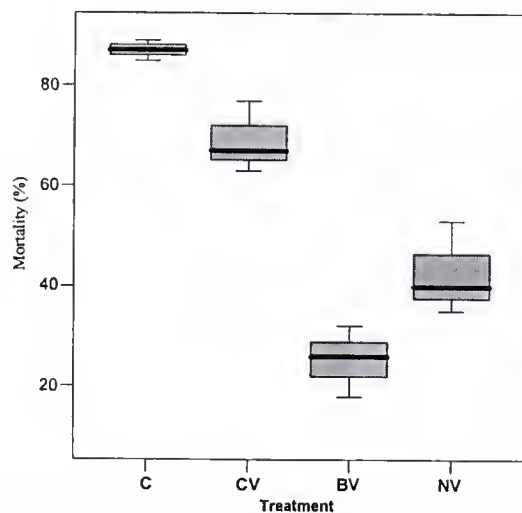


Figure 14-3. Average mortality rate (%) recorded in the second experiment with RTB sharks fed immunostimulants and immunostimulants free-diets for 12 days after challenge with *S. iniae* (1.5×10^5 bacteria/fish); 4 replicates were used per treatment and each tank was stocked with 25 fish. Boxes and bars respectively represent the distribution of 50% and 100% of data. The box plots gave an easier visualization of the range values of each treatment. Abbreviations: C= fish fed control diet, N= fish fed nucleotides, B= fish fed beta-glucans, V= vaccinated fish.

CHAPTER 15
EFFICACY OF VACCINATION OF ARTIFICIALLY, AND STRIP-SPAWNED
BROODSTOCK RED-TAIL BLACK SHARK (*Epalzeorhynchus bicolor*, FAM.
CYPRINIDAE) AGAINST *Streptococcus iniae*

15.1. Introduction

Because reproduction and immunity are two physiological activities that require high energy, a negative reciprocal relationship exists (Moshkin et al. 2003). It has been demonstrated in fish that administration of sex steroids can suppress immune functions such as phagocytic activity, lymphocyte mitosis, and antibody production (Hou et al. 1999; Yamaguchi et al. 2001). However, the effect of sex steroids can be more complicated, as observed in some studies where sex steroids caused immunostimulation (Paavonen et al. 1981). Additional proof of the interaction of sex hormones and immune systems is given by the presence of androgen and estrogen hormone receptors on leukocytes isolated from the head kidney and spleen of salmonids (Slater et al. 1995a; 1995b).

Any source of stress can influence reproduction and immune functions, generally with a negative outcome. Farmed fish are often subject to a variety of stressors, such as high stocking densities, handling, and confinement, which result in a decrease in growth rate and reproduction fitness. Stress can affect gamete development and quality, as well as egg and larval survival and development (Pankhurst and Van der Kraak, 1997). In largemouth bass, stressors have a negative effect on reproduction (Ostrand et al. 2004).

Handling during artificial spawning is a source of stress. Stressors registered by the brain cause the activation of the hypothalamus-pituitary axis. The hypothalamus

adrenal corticotropin hormone (ACTH). The hypothalamus also stimulates the chromaffin neurons primarily scattered through the kidney and the walls of the posterior cardinal vein, to release catecholamines in the blood circulation. ACTH stimulates chromaffin cells in the head kidney to release cortisol in the blood. Catecholamines and cortisol affect gills, anterior kidney, spleen, thymus, liver, muscle and the immune system. Catecholamines are involved in the initial response of the body to stress. They cause an increase of the basal metabolic rate, an increase of blood flow and pressure, and an increase in glycogenolysis to make glucose readily available to the body (Pickering, 1998). Cortisol and, in general, glucocorticoids are involved in the second phase of the response to stress: the resistance phase. Glucocorticoids stimulate a mobilization of the energy reserves to sustain the response of fish to stressful situations causing an increase of amino acids and lipid catabolism, and a decrease of glucose utilization by the muscle (Sumpter, 1997). Stressors can cause an osmotic imbalance due to ion leakage through cell junctions caused by an increase of blood flow in the gills and kidney (McDonald and Milligan, 1997).

Stress can also influence several immune functions (Schreck et al. 2001). ACTH can suppress the immune response to both T-dependent and T-independent antigens, lymphokines and IFN-gamma production by T-cells, Igs synthesis and macrophage activation by IFN-gamma (Blalock, 1989). Norepinephrine inhibits T-cell response, anti-viral immunity and natural killer cell activity (Sanders et al. 1997). In fish as in mammals, cortisol can cause a depression of circulating lymphocytes, antibody production, an inhibition of immune cell proliferation and growth, lymphokine production, macrophage phagocytic activity, bactericidal leukocyte activity, and

leukocyte distribution (Peter and Schreck, 1985). Glucocorticoids have a general immunosuppressive effect, as observed in experiments where injection of plasma from operated rats into normal animals caused a suppression of the activity of splenocytes, leukocytes and purified NK (Shakhar and Blumenfeld, 2003). Additional evidence confirms the role of glucocorticoids (corticosterone) in these mechanisms: corticosteroid receptor antagonist or corticosteroid synthesis inhibitor blocks suppression of NK cytotoxic activity, and the postoperative time course of plasma corticosterone levels parallels the inhibition of NK cells. However, glucocorticoids may not be the only cause of immunosuppression. For example, stress-induced elevations in glucocorticoid levels can occur in the absence of detectable changes in immune functions, and stress-induced immune alterations can occur in the absence of a detectable, or perhaps biologically meaningful, change in glucocorticoid levels (Moynihan, 2003). Other mechanisms and more complex pathways are also involved during stress, as shown from studies on adrenalectomized or hypophysectomized animals (Moynihan, 2003; Panerai et al. 1997; Shakhar and Blumenfeld, 2003). The effect of stress on the immune system is also influenced by several variables such as the nature of the stressor, acute versus chronic stress, the immune parameters examined, the experimental subject age, sex and genome. For example, acute stress enhanced the delayed type hypersensitivity (DTH) response in the rat, whereas DTH was suppressed with chronic stress (Dhabhar and McEwen, 1997). In some studies in mammals, stress affected only Th1 or Th2 cells suggesting that different neuroendocrine responses trigger changes in one, but not necessarily in both types of Th cells (Moynihan, 2003).

Even brief periods of stress can have negative effects. Handling of juvenile Chinook salmon for 30-60 seconds caused a severe suppression in the number of antibody-producing cells; but the disease resistance of the fish returned to normal levels the following day (Maule et al. 1989). Studies on alteration of the Na^+/Cl^- concentrations revealed that the recovery speed is dependent on the degree and type of stress, but generally after brief periods of stress fish recover totally within 24 h (Postlethwaite and McDonald, 1995). If stress stimuli have a brief duration, plasma catecholamines fall very rapidly (minutes), but, if the stress is more prolonged, plasma catecholamine levels can remain high for several days (Sumpter, 1997).

Negative feedback loops are present to regulate cortisol and catecholamine actions. In some loops the hormone directly regulates its own secretion; in others, the final product of a cascade acts as an inhibitor of the first step of the cascade. For example, cortisol inhibits CRH and ACTH release (Sumpter, 1997). Cortisol also causes an underregulation of the beta-adrenergic receptors. Catecholamine metabolism and clearance rate is stimulated during high levels of stress (Gampller and Boutilier, 1994). Fish can also control the sensitivity of target tissues by down-regulating cortisol receptor number and affinity (Pottinger et al. 1994), and decreasing the sensitivity of interrenal tissue to ACTH stimulation (Balm and Pottinger, 1995).

The results of some research on mammals (Dhabhar et al. 1995; Dhabhar and McEwen, 1996; May, 1996) suggest that, while certain aspects of immunity are depressed during stress, those at the periphery of the animal are enhanced, thus facilitating immune defense at the site of antigen entry into the organism. For example, stress decreases the number and percentage of lymphocytes and increases the number and

percentage of neutrophils in the body periphery, and the stress-induced changes are rapidly reversed on cessation of stress.

The main objective of this study was to determine if vaccines are effective in protecting broodstock fish against bacterial infections resulting from the stress caused by artificial spawning. We chose red-tail black sharks (*Epalzeorhynchus bicolor*, fam. Cyprinidae) (RTB shark) as a model because they are an economically important species for the Florida ornamental fish industry, and because after spawning these fish, some farmers may observe high mortality rates (> 80%) due to *Streptococcus iniae* infections. Two sets of experiments were designed. In the first two experiments, fish were vaccinated and spawned on an ornamental fish farm to mimic the normal spawning protocol. In the third experiment, fish were vaccinated on the farm, but at the moment of spawning they were moved into our laboratory where they were simultaneously spawned and challenged with *S. iniae*. Another goal of our research was to determine approximately how long the protection level conferred by vaccination would last, and for this purpose a fourth experiment was conducted. However, due to the lack of available ponds on the farm and the economic value of the broodstock, fish were challenged by *S. iniae* only 8 months after vaccination.

15.2. Materials and Methods

Experimental animals and system. The research was conducted in the Tropical Aquaculture Laboratory, University of Florida (Ruskin, FL), and in an ornamental fish farm in Hillsborough county (FL). Adult RTB shark (average weight 22.1 ± 5.9 g, average total length 12 ± 0.9 cm) were obtained from the same fish farm. At the farm,

fish were kept in a flow through system in 3,000l vats or in 26,500 L ponds. In the laboratory fish were kept in recirculating systems with 25 tanks of 38-L volume.

In all experiments in the laboratory, water temperature, ammonia, and nitrite were measured daily; alkalinity, hardness and pH once a week. At the farm all water parameters were measured weekly. The water in the laboratory was maintained at $25.5 \pm 0.6^{\circ}\text{C}$ and in the vats at the farm at $26 \pm 1^{\circ}\text{C}$. Water ammonia and nitrite concentrations were always close to 0 (mg/L); in the laboratory alkalinity and hardness were 110 ± 10 mg/L and 115 ± 15 mg/L respectively; pH was 7.5; similar values were measured in the farm.

Bacterial strain. Details of the bacterial isolation and batch broth culture were reported previously (Chapter 9).

Vaccine production. Details of the vaccines production were reported previously (Chapter 10).

First and second experimental design. The purpose of these two experiments was to determine if vaccines are effective in protecting broodstock fish against *S. iniae* infections resulting from the stress caused by artificial spawning, and both experiments were conducted at the farm to mimic the normal spawning protocol. The same protocol was followed in both experiments.

RTB broodstock fish were harvested from a pond and moved into vats inside a greenhouse at the farm. Due to space limitations, only three vats for treatments were used and stocked with 60 fish/vat. Fish were acclimatized for a week and then vaccinated with an intracoelomic injection of 0.1 mL of the aluminium or oil formulation. Another group served as a control.

After 21 days, fish were injected with a dose of 0.5 mL/kg body weight, and the following day artificially spawned. For the artificial spawning, fish were first anaesthetised with 100mg/L MS-222, dried with a paper towel and then stripped. Mortality was recorded for 12 days after spawning.

Third experimental design. The purpose of this experiment was also to determine if vaccines are effective in protecting broodstock fish against *S. iniae* infections after artificial spawning, but in this experiment at the moment of spawning, the fish were moved into our laboratory where they were simultaneously spawned and challenged with *S. iniae*.

RTB broodstock fish were harvested from a pond and moved into vats inside a greenhouse at the farm. Due to space limitations, only three vats were used for each treatment and stocked with 60 fish/ vat. Fish were acclimatized for a week and then vaccinated with an intracoelomic injection of 0.1 mL of the aluminium or oil formulation. Another group served as a control.

After 21 days, fish were moved from the farm into the systems in the laboratory. Each tank was stocked with 12 fish. After 1 day of acclimation, fish were spawned using the same protocol described in the first series of experiments; ovaprim was not injected in this experiment to avoid another source of stress. Before spawning, fish from each group (nonvaccinated fish, fish vaccinated with the aluminium or oil formulation) were divided in 3 subsets of treatments: (1) fish unspawned and challenged with *S. iniae* by injection (C, AL, OL), (2) fish spawned and challenged with *S. iniae* by injection (SPBAC, SPBAAL, SPBAOL), (3) fish spawned and challenged with *S. iniae* by bath (SPC, SPAL, SPOL). Fish were challenged with an intracoelomic injection of 1.5×10^5 *S. iniae*

cells/fish or by bath turning off the UV lights so that the fish were left exposed to *S. iniae* shed by fish in the other treatments. Four replicates were used for each treatment.

Mortality was recorded for 10 days after spawning and during this period, dead fish were removed 3 times at day. Brain and kidney cultures of 70-80% of the moribund or dead fish were taken for microbiological testing in an attempt to verify the cause of mortality.

Fourth experimental design. The purpose of this experiment was to determine approximately how long the protection level conferred by vaccination would last.

RTB broodstock fish were harvested from a pond and moved into vats inside a greenhouse at the farm and vaccinated with the basic, or aluminium or oil formulations following the protocol of the previous experiment designs.

After 21 days from vaccination, fish were not spawned but moved into 3 outdoor ponds in the farm. Due to pond availability at the farm, it was not possible to stock a pond with nonvaccinated fish, and, for the same reason, fish could not be kept in ponds for a period longer than 8 months.

RTB sharks were harvested from the ponds after 8 months and moved to vats inside the greenhouse. At the same time, 3 groups of new broodstock fish were harvested from another pond, and moved to the same greenhouse. Each vat was stocked with 60 fish. After 1 week of acclimation they were vaccinated by intracoelomic injection of 0.1 mL of the aluminium or oil formulation.

After 21 days, all fish (old and new groups of broodstock) were moved to the systems inside the laboratory. Each tank was stocked with 12 fish. After one day of acclimation, all fish were challenged with 1.5×10^5 *S. iniae* cells/fish by an intracoelomic injection of 0.05 mL bacterial culture. Four replicates were used for each treatment.

Mortality was recorded for 10 days after spawning and, during this period, dead fish were removed 3 times at day. Brain and kidney cultures of 70-80% moribund or dead fish were taken for microbiological testing in an attempt to verify the cause of mortality.

Statistical analysis. ANOVA analysis and Tukey's post hoc test were run for each experiment using the statistical program SPSS 12.0 (SPSS Inc., Chicago, IL). An asin(square root) transformation was performed on the mortality data expressed as percentage. The results of the ANOVA analyses were considered significant at a $p\text{-value} < 0.01$; then the Tukey's test was considered at an $\alpha = 0.05$. Relative percent survival (RPS) was calculated with the formula:

$$\text{RPS} = \left[1 - \frac{\text{treatment mortality (\%)}}{\text{control mortality (\%)}} \right] * 100$$

15.3. Results

In the first two experiments conducted at the farm, low mortality ($< 5\%$) was recorded after spawning and for the following 14 days and there was no significant difference between vaccinated and nonvaccinated fish.

In the third experiment (Figure 15-1 and Table 15-1) the mortality after bacterial challenge of spawned fish vaccinated with the aluminium ($13 \pm 23\%$ for SPBAAL and $4 \pm 7\%$ for SPAL) or oil formulation ($25 \pm 22\%$ for SPBAOL and $23 \pm 30\%$ for SPOL) was significantly lower than control groups ($100 \pm 0\%$ for both SPBAC and SPC). There was also no significant difference in mortality between spawned and unspawned fish ($20 \pm 22\%$ for AL and $46 \pm 18\%$ for OL).

In the fourth experiment (Table 15-2, Figure 15-2) the mortality of fish vaccinated 8 months before the bacterial challenge with the aluminium (ALOLD, $72 \pm 17\%$) or oil

(OLOLD, $71 \pm 9\%$) formulation was significantly lower than for the control group (C, $100 \pm 0\%$), but the mortality of fish vaccinated with the basic formulation (WOLD, $90 \pm 8\%$) was not significantly different from the control. The mortality of fish vaccinated 8 months before the bacterial challenge was significantly higher than the mortality of fish vaccinated only from 1 month before challenge ($18 \pm 19\%$ and $18 \pm 23\%$ respectively for the aluminium and oil formulations).

15.4. Discussion

In our trials at the farm we failed to observe mass mortality due to *S. iniae* after spawning in vaccinated and nonvaccinated fish, as reported previously by the commercial fish farmers. However, for a few years, at least one farm has begun to use antibiotics (specifically erythromycin) as part of a preventative protocol post spawning, and they now report a decrease of *S. iniae* outbreaks. The use of erythromycin may have caused a decrease in the bacterial presence on the farm, but this use may result in selection for resistant bacterial strains. This possible result highlights the importance of vaccine use in lieu of antibiotics, where feasible.

The results of the second series of experiments demonstrated that both vaccine formulations (aluminium and oil) provided some protection even during artificial strip spawning. This experiment was planned because for obvious reasons, fish could not be challenged on the farm. Fish were acclimatized to the new system in our laboratory only for one day before spawning-challenge because of the relative size of the fish compared to the tank volume and the aggressive behavior of sharks that would have been amplified in such a high density situation. Spawned fish were challenged by intracoelomic injection to be sure they were exposed to a sufficient dose of bacteria to cause the mortality of at

least 70% of nonvaccinated fish. This bacterial concentration was determined in previous experiments (Chapter 9). A group of fish was challenged by bath to try to simulate the normal infection route. The mortality rates between fish challenged by the two ways were similar, but the mortality in fish challenged by bath was delayed a few days. The wide range of the standard deviation was due to the relatively low numbers of fish per tank, but because of tank volume and fish size, no more fish could be stocked, and due to the economic value of the broodstock, no more replicates could be used.

The result of duration of the vaccine protection over time showed that a certain level of protection is still present after 8 months. Other studies have investigated the duration of vaccine protection in time. For example, in trout vaccinated against *S. iniae* the protection lasted at least four months and there was a progressive decrease in antibody titers until a minimal detectable level was reached after 6 months (Bercovier et al. 1997). Atlantic salmon vaccinated against furunculosis were protected for 6 months (Midtlyng et al. 1996a).

The results of our research are useful for developing a vaccination protocol for broodstock red-tail black sharks where they are vaccinated once a month before the beginning of the spawning season. A booster injection of vaccine a few weeks after the first vaccination might help to increase the efficacy of the vaccine. This protocol would decrease the stress on the fish from that of the actual preventative protocol consisting of injecting antibiotics in broodstock three times after each spawning event. In a spawning season each brood fish can be spawned up to four times. Furthermore, vaccination decreases the possibility of selecting for resistant strains of bacteria which may occur with continual use of antibiotics.

Even if the protection level conferred by the vaccine does not remain high for a long time, these are important results because they represent a forward step in developing a vaccine for sharks. Low efficacy may be observed in the first attempt to develop new vaccines, but the results of the first attempts are important to set a baseline level of protection and to allow improvement and development of future vaccines (Mitchell, 1999). It is known that especially with killed bacterial vaccines (Gaskin, J., personal communication) booster injections are required to increase antibody titers. In Atlantic salmon booster injections of a vaccine against *Piscirickettsia salmonis* increased the antibody response and resistance (O'Dowd et al. 1999).

As observed in previous experiments (Chapter 10), and in accordance with reports in the literature (Horne et al. 1984; Midtlyng, 1996), adjuvants (aluminum or oil) can significantly increase vaccine efficacy even after several months, as we observed in the last experiment. In accordance with the results of our other previous experiments (Chapters 11 and 12) the aluminium formulation was more effective at protecting the fish than the oil formulation, although in this study this difference was not significant. Interestingly in salmonids, the oil formulation conferred a higher protection level than the aluminum formulation (Midtlyng et al. 1996ab; Midtlyng, 1996; Nordmo and Ramstad, 1997).

15.5. Conclusions

The results of this research demonstrated that even in a highly stressful situation such as is found in artificial strip spawning, vaccines are still efficient in protecting fish against bacterial diseases. These results are of a particular importance for designing vaccination protocols for food and ornamental fish. Antibiotics are used during artificial

spawning on some farms, and this methodology causes increased stress to the broodstock due to the frequent handling and antibiotic injections. Furthermore there is an increased risk of selecting resistant strains of bacteria in the environment. For this reason it is important to promote vaccination protocols and to demonstrate their efficacy to commercial fish farmers. Furthermore, vaccination is also cheaper than the use of antibiotics because only 1-2 injections during the spawning season are required compared to the several injections required after each spawning. Labor expenses, in addition to drug expenses, are decreased.

Table 15-1. Total average mortality (%) recorded in RTB broodstock for 14 days after challenge with *S. iniae* (see Materials and Methods for details). In the last column are reported the level of the significant difference among treatments as resulted from the Tukey's test.

Treatment	Average Mortality %±SD	RPS %	Significance
C	100 ± 0	0	a
OL	46 ± 18	54	b
AL	20 ± 22	80	b
SPBAC	100 ± 0	0	a
SPBAOL	25 ± 22	75	b
SPBAAL	13 ± 23	87	b
SPC	85 ± 17	0	a
SPOL	23 ± 30	73	b
SPAL	4 ± 7	95	b

($p = 6.3 \times 10^{-6}$, $\alpha = 0.05$); 4 replicates were used per treatment and each tank was stocked with 12 fish. Abbreviations: RPS= relative percent survival, C= control, OL= fish vaccinated with the oil formulation, AL= fish vaccinated with aluminium formulation, SPBA= fish artificially spawned and injected with *S. iniae*, SP= fish artificially spawned and challenged with *S. iniae* by bath.

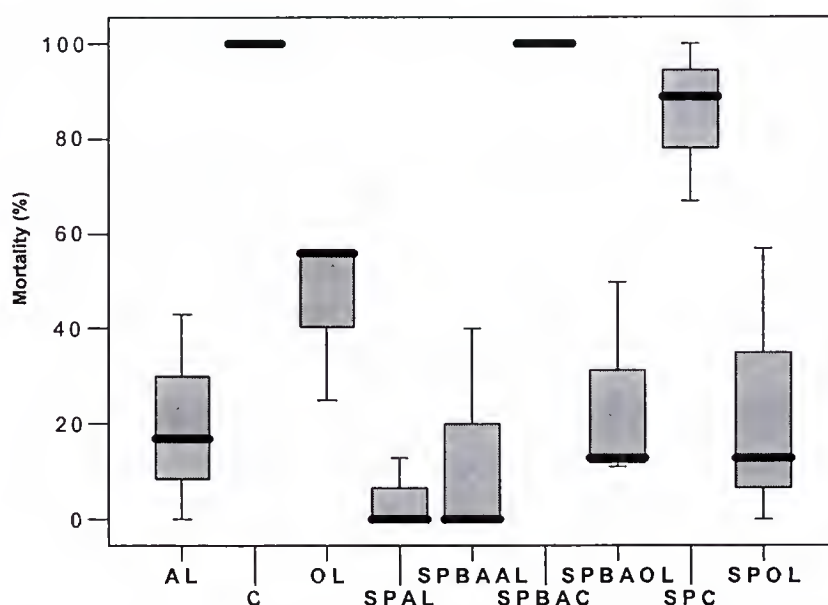


Figure 15-1. Box plots of the total mortality (%) recorded in RTB broodstock for 14 days after challenge with *S. iniae* (see Materials and Methods for details); 4 replicates were used per treatment and each tank was stocked with 12 fish. Boxes and bars respectively represent the distribution of 50% and 100% of data. Abbreviations: C= control, OL= fish vaccinated with the oil formulation, AL= fish vaccinated with aluminium formulation, SPBA= fish artificially spawned and injected with *S. iniae*, SP= fish artificially spawned and challenged with *S. iniae* by bath.

Table 15-2. Total average mortality (%) recorded in RTB broodstock vaccinated 8 months before the experiment, for 14 days after the challenge with *S. iniae* (see Materials and Methods) for details). In the last column are reported the significant difference among treatments as resulted from the Tukey's test.

Treatment	Average Mortality %±SD	RPS %	Significance
C	100 ± 0	0	a
OL	18 ± 23	82	c
AL	18 ± 19	82	c
WOLD	90 ± 8	10	a,b
OLOLD	71 ± 9	29	b
ALOLD	72 ± 17	28	b

($p = 1.7 \times 10^{-5}$, $\alpha = 0.05$); 4 replicates were used per treatment, and each tank was stocked with 12 fish. Abbreviations: RPS= relative percent survival, C= control, OL= fish vaccinated with the oil formulation, AL= fish vaccinated with the aluminum formulation, OLD= fish vaccinated 8 months before the experiment, W= fish vaccinated with the base formulation.

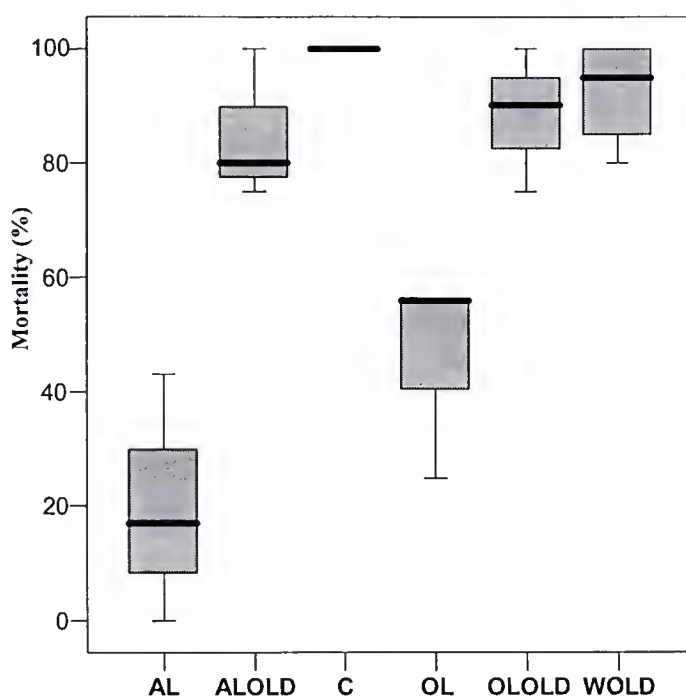


Figure 15-2. Box plots of the total mortality (%) recorded in RTB broodstock vaccinated from 8 months, for 14 days after challenge with *S. iniae* (see Materials and Methods for details); 4 replicates were used per treatment and each tank was stocked with 15 fish. Boxes and bars respectively represent the distribution of 50% and 100% of data. Abbreviations: C= control, OL= fish vaccinated with the oil formulation, AL= fish vaccinated with aluminium formulation, OLD= fish vaccinated 8 months before the experiment, W= fish vaccinated with the base formulation.

CHAPTER 16 AGGLUTINATION AND ELISA TESTS

16.1. Introduction

Agglutination and ELISA tests have been used in several experimental infection and vaccine studies (Akhlaghi et al. 1996; Eldar et al. 1997; Evans et al. 2000b; Klesius et al. 2000; Sakai et al. 1987; Shelby et al. 2002a) to detect antibody production to specific pathogens in fish. Monoclonal antibodies (Mab) against fish immunoglobulins (Igs) have been produced for several species of fish; for example, Mab have been developed against Nile tilapia (*Oreochromis niloticus*) Igs to detect the humoral response to *S. iniae*. (Shelby et al. 2001; 2002b). The goal of this study was to detect shark specific antibodies against *S. iniae* by use of agglutination and ELISA tests. The agglutination and ELISA systems were first investigated using serum from koi carp (*Cyprinus carpio*).

16.2. Materials and Methods

Experimental animals. Blood was collected from vaccinated (with aluminum or oil vaccine formulations) and unvaccinated adult red-tail black sharks (RTB shark) (21.2 ± 5.7 g WT, 12 ± 0.8 cm TL) and adult rainbow sharks (20.4 ± 4.2 g WT, 10.2 ± 0.5 cm TL) used in previous experiments (Chapters 12 and 15). Blood was collected from 8 fish per treatment. Fish were bled 3 weeks after vaccination; survival fish to the challenge with *S. iniae*, were bled 12 days after the bacterial challenge.

Blood samples from 4 different groups of koi carp (vaccinated with the aluminum or oil formulations but not challenged; unvaccinated fish challenged with *S. iniae*; and control group) were collected to develop the ELISA and agglutination tests. For each of

the four treatment groups, 12 koi carps were bled. Vaccinated koi were intracoelomically injected with 0.8 mL of the aluminum or oil formulations, and bled after 3 and 5 weeks. Challenged koi were intracoelomically injected with 0.5 mL of *S. iniae* culture corresponding to 1.5×10^5 bacteria cells. After 3 weeks, these koi were bled. No mortality was observed in the period after the bacterial challenge.

Fish in each group were bled via the caudal vein to yield as much blood as possible in sharks (less than 0.1 mL) and in koi (0.15 mL) which was then pooled. Serum was removed after centrifugation of blood samples (1200 g for 15 minutes) and stored at -70°C .

Slide Agglutination test. *Streptococcus iniae* was grown in brain heart infusion broth for 48 h at 37°C . The bacteria were pelleted by centrifugation (1200 g for 30 minutes), the supernatant broth was discarded, and the bacteria were re-centrifuged three more times with rinses of isotonic phosphate-buffered saline (PBS). The bacterial pellet was re-suspended in sufficient PBS to form a highly concentrated uniformly opaque suspension. A drop of this suspension was mixed with a drop of undiluted koi serum (using sera from both immune and non-immune koi) on a glass slide and incubated for several minutes at room temperature. A visible clumping effect was considered a positive reaction, and a uniformly opaque mixture was considered to be a negative reaction.

ELISAs. An indirect ELISA was run to determine if the mouse monoclonal anti-carp Igs obtained from Dr. Jan H. W. M. Rombout (Cell Biology and Immunology Group, Wageningen University, The Netherlands) reacted with koi and shark antibodies; and another indirect ELISA was run to detect koi and shark anti-*S. iniae* Igs.

First indirect ELISA. The first column of plastic microtiter plates (8 rows and 12 columns, Becton-Dickison, Lincoln Park, New Jersey) was coated with koi or RTB shark serum at dilutions between 1/10 and 1/100 from the control group (Table 16-1), and sequential dilutions in carbonate-bicarbonate buffer (pH = 9,6) were done in the following columns. The plates were left overnight at 4°C and then washed 3 times with PBS+0.05 tween-20 (PBST). One hundred µL of blocking buffer (5% nonfat dry milk in TTBS) was added to each well and the plates were incubated for 30 minute at 37°C. Plates were washed with PBST as before. Mouse monoclonal anti-carp Igs were diluted at 1/100 and 100 µL were added in each well, then the plates were incubated for 30 minute at 37°C. Plates were washed with PBST as before. Horseradish peroxidase-labelled goat monoclonal anti-mouse IgG Pierce, Rockford, Illinois) was diluted at 1/200 and 100 µL were added in each well, then the plates were incubated for 30 minute at 37°C. Plates were washed 5 times with PBST, and 100 µL of peroxide/chromogenic substrate solution (TMB solution + peroxide solution (Pierce, Rockford, Illinois) was added to each well and incubated at room temperature. After 15-30 minutes plates were read with a spectrophotometer (Benchmark Plus, Bio-Ras Laboratory, Hercules, California).

Second indirect ELISA. When koi serum was analyzed, the first column of plates were coated with 1/10 dilution of (1) *S. iniae* broth culture in BHI, or (2) *S. iniae* BHI broth pellet, or (3) supernatant, or (4) *S. iniae* broth culture in TSI, or (5) sonicate *S. iniae* pellet, or (6) sonicate *S. iniae* supernatant (Table 16-2). The first column of control rows of plates coated with BHI (1-3) or TSI bacterial cultures (4) were coated with 1/10 dilution of sterile BHI or TSB media. Sequential dilutions in carbonate-bicarbonate

buffer (ph = 9,6) were done in the following columns. When shark serum was analyzed, plates were coated only with sonicated *S. iniae* supernatant at a dilution of 1/10. Cultures of *S. iniae* were incubated for 24 h at 37°C. Bacterial pellet and supernatant were obtained by centrifugation of the broth cultures at 12,000 g for 15 minutes. Pellets from broth cultures were sonicated (Sonicator Heat System, Ultrasonics, Inc., Plainview, New York) in 12 cycles of 30 second at 50% duty cycle. The resulting sonicate was centrifuged at 12,000 g for 15 minutes. The total protein concentration of the sonicate supernatant as determined by the bicinchoninic acid (BCA) method was 310.7 µg/mL.

After coating, plates were left overnight at 4°C and then washed 3 times with PBS+0.05 tween-20 (PBST). One hundred µL of blocking buffer (5% nonfat dry milk in TTBS) was added to each well, and the plates were incubated for 30 minute at 37°C. Plates were washed with PBST as before. One hundred µL of koi or shark serum was added at dilution 1/1, or 1/10, or 1/100, or 1/1000 in each well. Plates were incubated for 30 minute at 37°C, and then washed with PBST as before. Mouse monoclonal anti-carp Igs were diluted at 1/100 and 100 µL were added in each well, then the plates were incubated for 30 minute at 37°C. Plates were washed with PBST as before. Horseradish peroxidase-labelled goat anti-mouse IgM (Pierce, Rockford, Illinois) was diluted at 1/200 and 100 µL were added in each well, and the plates were incubated for 30 minute at 37°C. Plates were washed 5 times with PBST, and 100 µL of of peroxide/chromogenic substrate solution (TMB solution + peroxide solution. Pierce, Rockford, Illinois) was added to each well and incubated at room temperature. After 15-30 minutes, the plates were read with a spectrophotometer (Benchmark Plus, Bio-Ras Laboratory, Hercules, California).

16.3. Results

With the agglutination test was not possible to detect specific antibodies against *S. iniae* in koi or RTB shark, even with undiluted serum.

Koi Igs were detected with the indirect ELISA (Table 16-1). The strongest absorbance signal was obtained when wells were coated with koi serum dilutions between 1/1,000 and 1/16,000. With RTB shark serum the strongest absorbance signal was obtained with serum dilutions between 1/10 and 1/160.

In the second indirect ELISA (Table 16-2), positive absorbance signals were observed in all wells coated with *S. iniae* BHI or TSI broth cultures, or with supernatant or pellet from *S. iniae* BHI broth cultures when serum from vaccinated and challenged koi at dilution 1/10 or 1/100 was added, compared to no absorbance signal from serum of the control group. However, non-specific background reactivity, represented by random coloration, was observed in the control when plates were coated with *S. iniae* culture pellet. No difference in absorbance was observed between fish vaccinated with the aluminum and oil vaccines. The same level of absorbance that was detected when plates were coated with *S. iniae* BHI or TSI cultures was also observed in wells coated with sterile BHI or TSI when sera from vaccinated and challenged koi was added.

Positive absorbance signals were observed in wells coated with sonicate *S. iniae* pellet or supernatant at dilution 1/10 and 1/80 when sera from vaccinated and challenged koi at 1/10 dilution were added, and no absorbance signal was detected in wells corresponding to the serum of the control group. However, random non-specific background coloration was observed in all wells coated with sonicate bacterial pellet. No

difference in absorbance was observed between fish vaccinated with the aluminum and oil vaccines.

Positive absorbance signals were observed in wells coated with sonicate *S. iniae* supernatant when sera from vaccinated RTB and rainbow shark survivors of bacterial challenge were added, and no absorbance signal was detected when sera of vaccinated but not challenged RTB shark and of RTB and rainbow shark control groups were added. No difference in absorbance was observed between fish vaccinated with the aluminum and oil vaccines. Absorbance signals were observed only when undiluted RTB and rainbow shark sera were used.

16.4. Discussion

The results of the first set of ELISAs showed that the mouse Mab anti-carp Igs are still able to recognize and bind koi Igs. In the second set of ELISAs, when *S. iniae* cultures in BHI or TSI were used to coat the plates, it appears that specific koi Igs anti-*S. iniae* were detected because of the positive absorbance values observed for serum samples corresponding to vaccinated and challenged koi, and the negative absorbance observed in the control koi. In reality, in these samples not only koi Igs against *S. iniae* were detected, but also antibody against several components of the BHI and TSI media, as shown by the same level of absorbance detected when sera from vaccinated and challenged koi were added to wells coated with sterile BHI or TSI. Furthermore, the absorbance was higher when wells were coated with BHI media, a result expected because the *S. iniae* cultures used for making the vaccine and for the challenge were grown in BHI media. Specific koi Igs against *S. iniae* were detected when sonicate *S. iniae* cultures were coated to the plates.

RTB and rainbow sharks Igs were detected in the second indirect ELISA, even if at much lower dilutions than koi Igs, a result expected using mouse Mab anti-carp Igs. The fact that a positive reaction was observed with the ELISA test only in vaccinated sharks that survived bacterial challenge (so exposed twice to *S. iniae* antigens) is an indication that the mouse Mab anti-carp Igs might recognize something, but the affinity of the mouse Mab anti-carp Igs is too low for reliable detection. It can not be concluded that the negative animals (shark only vaccinated but not challenged and control group) are really negative, because the level of shark Igs against-*S. iniae* might have been too low to be detected, and it is well known that the immune response in wild type animals can vary strongly (Rombout personal communication).

16.5. Conclusions

The results of this study have shown that mouse Mab anti-carp Igs are able to recognize shark Igs, but with a much lower affinity compared to carp (koi) Igs. However, this heterologous recognition of shark Igs is too low to allow a quantitative measurement of the antibody response to pathogens. Several studies (Eldar et al. 1995b; Evans et al. 2000b; Lillehaug et al. 1993; Taylor et al. 2001) have failed to observe a correlation between protection given by vaccines and antibody titers. These results suggest the possibility that in fish the nonspecific immune system is mainly involved in fighting pathogens.

The results of this study also showed that ELISA has a higher sensitivity level in antibody detection than does the slide agglutination test, as has been observed in other studies (Shelby et al. 2001; 2002b). For this reason ELISA would be the superior

technique for monitoring humoral immune responses to vaccination, provided shark specific anti-Igs were available as reagents

Table 16-1. Range of koi and RTB shark serum dilutions tested in the indirect ELISA. In the second column are reported the range of serum dilution corresponding at the higher absorbance observed.

Species	Range serum dilution tested	Absorbance observed for serum dilution
Koi carp (control) <i>Cyprinus carpio</i>	1/10- 1/2,048,000	1/1,000- 1/16,000
RTB shark (control) <i>Epalzeorhynchus bicolor</i>	1/10- 1/204,800	1/10- 1/160

Table 16-2. Coating agents used in the indirect ELISA to detect koi or RTB or rainbow sharks Igs anti *S. iniae*, and coating agent dilutions that gave positive reactions. Koi serum at 1/10 dilution and sharks serum at 1/1 dilution was added in each well. No difference in absorbance was observed between koi or sharks vaccinated with the aluminum or oil formulation, for this reason in the table is not specified the vaccine formulation.

Plate coating agent <i>S. iniae</i> culture	Absorbance observed for plate coating agent dilution		
Koi carp (<i>C. carpio</i>) Serum dilution 1/10	Control	Bacterial challenged	Vaccinated
BHI broth	negative	1/10- 1/24,080	1/10- 1/24,080
BHI broth supernatant	negative	1/10- 1/24,080	1/10- 1/24,080
BHI broth pellet	background noise	1/10- 1/24,080	1/10- 1/24,080
sterile BHI	negative	1/10- 1/24,080	1/10- 1/24,080
TSI broth	negative	1/10- 1/24,080	1/10- 1/24,080
TSI broth supernatant	negative	1/10- 1/24,080	1/10- 1/24,080
TSI broth pellet	background noise	1/10- 1/24,080	1/10- 1/24,080
sterile TSI	negative	1/10- 1/24,080	1/10- 1/24,080
Sonicate pellet	background noise	1/10- 1/80	1/10- 1/80
Sonicate supernatant	negative	1/10- 1/80	1/10- 1/80
RTB shark (<i>E. bicolor</i>) Serum dilution 1/1	Control	Vaccinated and challenged	Vaccinated
Sonicate supernatant	negative	1/10	negative
Rainbow shark (<i>E. erythrurus</i>) Serum dilution 1/1	Control	Vaccinated and challenged	
Sonicate supernatant	negative	1/10	negative

CHAPTER 17

CONCLUSION

The results of this research have allowed the development of challenge models and vaccine efficacy tests for two ornamental fish, which may also be applied for future studies on new vaccine for other species of ornamental fish. As it has been described previously, low efficacy may be observed in the first attempts to develop new vaccines, but the results of the first attempts are important in establishing a baseline level of protection and for allowing improvement and development of future vaccines. This research has also been useful in outlining and partially resolving some of the problems in developing new vaccines for ornamental fish, such as the severe side effects observed after the injection of the vaccines due to the small size of juvenile sharks.

This research provides a good example of how the protection level conferred by vaccines can be variable. Especially in the field, there is often a misunderstanding of what is meant by “vaccine efficacy” and the fact that the results of vaccination procedures can be uncertain. It is not uncommon that a group of vaccinated fish has the same mortality rate as control fish. Vaccines help to prevent diseases and reduce outbreak severity, but do not prevent infections. The effect of vaccination is usually observed over a long period of time and consists of decreasing or eliminating disease incidence. For this reason vaccine efficacy is more complicated to determine than disease prevention. Vaccines can also increase resistance to other disease or stress situations, and indirectly

improve growth rate and feed efficiency, so as to make their use economically effective even if there is no decrease in mortality. The efficacy of vaccines is also influenced by many factors (genetic, nutrition quality, stress, water temperature, handling, etc.), and most of them are either uncontrollable or underestimated. For this reason although great differences in results might be seen among facilities, this does not necessarily imply that the vaccination program is not efficient.

In this research we demonstrated the efficacy of a vaccine against *S. iniae* when adjuvants (aluminum salt or oil) were added to the basic formulation. As reported many times in the literature, adjuvants are very effective in increasing the immunostimulatory capability of vaccines due to their mechanisms of action. Unfortunately, in our studies the data indicated that only the injection route of administration was efficient in providing protection to the fish, but, for obvious reasons, this route is not very economically feasible for juvenile fish. Construction of an automated vaccine delivery system or improvement of oral delivery of vaccines using microcapsule could make vaccination by injection in juvenile fish economically feasible for the ornamental fish industry. However, in juveniles, as in adults, immunostimulants might be used to increase the resistance to disease. This research has demonstrated the positive effect on disease resistance of administration of immunostimulants to fish. The results of the experiments with broodstock fish demonstrated how vaccines are very effective in protecting fish even in a highly stressful situation, such as is found in artificial strip spawning. For this reason vaccination programs are advisable for broodstock fish.

Under the conditions of this research, we observed a difference in vaccine efficacy among red-tail black shark, rainbow shark, and blue shark. This is one of the

more interesting findings of this research. Disease resistance due to genetic variation is an important factor that should be considered for developing successful breeding programs. The selection of strains of fish less susceptible to disease results in an increase of biomass production and in a decrease of production expenses. However, because the strain of *S. iniae* used in this research was originally isolated from rainbow, this might be another possible contributing factor to the difference in disease resistance between RTB and rainbow shark.

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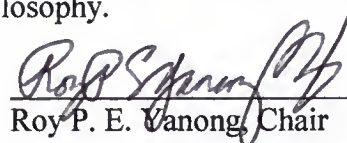
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BIOGRAPHICAL SKETCH

Riccardo Russo was born December 17, 1973, in Rome, Italy. In 1998, he received his *laurea* degree (comparable to a Master of Science) with honors in biological sciences, from the University of Rome Tor Vergata. After graduation, he worked at the University of Tor Vergata in fish parasitology and ecology. In 1999, after being awarded a scholarship from the same University, Riccardo moved to Fort Pierce, Florida, for practical training in aquaculture, at the Harbor Branch Oceanographic Institution, where he worked in fish, shrimp, and clam reproduction, hatchery, and grow-out. There, in addition, he was awarded a scholarship for a summer internship in the Biomedical Research Department.

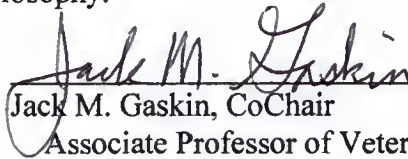
In spring 2001, Riccardo began his Ph.D. at the University of Florida, in the Department of Fisheries and Aquatic Sciences, studying fish health and vaccinology, with a minor in animal nutrition. After completion of his studies, Riccardo plans to enter a postdoctoral program in fish vaccinology or work in the fish health industry.

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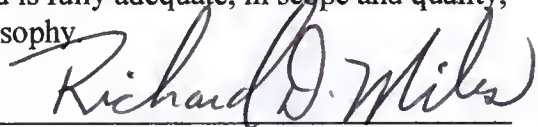
Roy P. E. Vanong, Chair
Associate Professor of Fisheries and
Aquatic Sciences

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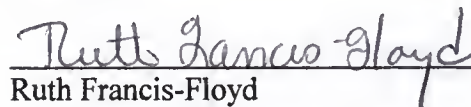
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Richard D. Miles
Professor of Animal Sciences

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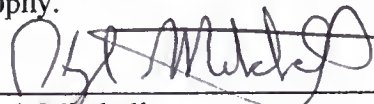
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John Tucker Jr.
Special Member

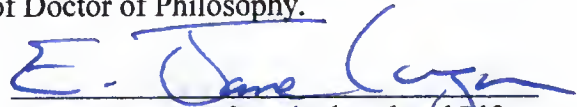
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Hugh Mitchell
Special Member

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 2004



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